

IDENTIFICATION OF A NEW COMPONENT IN THE MURINE Ia MOLECULAR COMPLEX*

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The class II major histocompatibility antigens, termed Ia in the mouse, are genetically polymorphic glycoprotein heterodimers. Although recent evidence suggests that these glycoproteins are responsible for mediating immune response (Ir)¹ gene effects (1, 2), the mechanism(s) through which Ia antigens regulate Ir gene function is still not understood. One approach designed to address this issue is to obtain detailed information on the structure of these antigens to determine what structural features can account for the observed biological properties of Ia. Newly developed techniques in molecular biology have allowed rapid advances in our knowledge of the primary structure of the class II gene products (3–5). This methodology does not, however, provide information on the structural features of Ia that are generated posttranslationally, features that may play a role in Ir gene function or in regulation of Ia antigen expression.

One of the notable properties of Ia molecules, detectable by isoelectric focusing analysis, is a high degree of charge heterogeneity (6). Generally, charge heterogeneity of glycoproteins is attributed to variable amounts of sialic acid on the terminal sugars of the carbohydrate moieties of the glycoproteins. However, neuraminidase treatment of Ia does not totally eliminate the charge heterogeneity of Ia α and β chains (7, 8), indicating either that some sialic acid residues are not sensitive to the treatment or that other charged residues are present in variable amounts.

It has recently been discovered that mammalian glycoproteins can be sulfated and thus sulfation can be included among the modifications conferring charge on these molecules. Sulfate has been identified on both O- and N-linked carbohydrate groups of glycoproteins (9–12), as well as in direct linkage to amino acid residues (13). We began the following experiments to determine whether some of the charge heterogeneity of Ia could be due to posttranslational addition of sulfate to Ia α or β chains. We were unable to detect sulfation of conventional

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¹ *Abbreviations used in this paper:* dFCS, dialyzed fetal calf serum; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; I_i, associated invariant glycoproteins; Ir, immune response; MHC, major histocompatibility complex; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PSN, phosphate-buffered saline containing 0.25% NP-40; SaCl, Staphylococcus aureus Cowan Strain I; SDS, sodium dodecyl sulfate; TLCK, N- α -tosyl-L-lysine chloromethyl ketone; TPCCK, L-1-tosylamide-2-phenylethyl chloromethyl ketone.

Ia glycoproteins, but in the course of these experiments we did detect a heretofore undescribed component of the Ia antigen complex.

Materials and Methods

Mice. Mice were obtained from the animal facility of the Jewish Hospital of St. Louis, St. Louis, MO, and were used at 8–12 wk of age.

Antibodies. A.TH anti A.TL (αI^k) and (129XA.TL)F₁ α A.AL (αK^k) alloantisera were produced and supplied by Dr. D. Shreffler, Washington University, St. Louis, MO. The monoclonal antibody 10-2.16 (anti-Ia.17, used as an anti I-A^k reagent) was obtained from the 10-2.16 cell line (14), supplied by the Salk Institute. MKD6 (anti I-A^d) (15) was originally obtained from Drs. P. Marrack and J. Kappler of the National Jewish Hospital, Denver, CO. The Do 3 monoclonal antibody was developed by Drs. M. Nahm and V. Hauptfeld, Washington University, St. Louis, MO (16). It reacts with the Ia.7 specificity in cytotoxicity assays,² and it precipitates I-E glycoproteins (S. Cullen, unpublished observations). In-1, a rat anti-mouse invariant chain monoclonal antibody (17), was produced and generously provided by G. Hammerling, Deutsches Krebsforschungszentrum, Heidelberg, Germany. Rabbit anti-rat Ig serum was purchased from Cappel Laboratories, West Chester, PA.

Biosynthetic Labeling. A spleen cell suspension was prepared and the splenocytes were washed twice in phosphate-buffered saline (PBS, pH 7.2) containing 1% dialyzed fetal calf serum (dFCS). Before biosynthetic labeling, cells were preincubated in precursor-deficient medium for 30–45 min at 37°C, then pelleted by centrifugation and resuspended in medium containing the radioactive precursor. The medium used for [³⁵S]sulfate labeling was Earle's balanced salt solution (with MgCl₂ substituted for MgSO₄) supplemented with amino acids and vitamins from an RPMI Select-amine kit (Gibco Laboratories, Grand Island, NY), glutamine, 10 mM Hepes, 5.0% dFCS, and aqueous ³⁵SO₄ (1,100–1,300 Ci/mmole; Amersham Corp., Arlington Heights, IL at 200–500 μ Ci/ml. For [³⁵S]methionine or [³H]leucine labeling, the medium used was RPMI 1640 lacking methionine or leucine, supplemented as above with glutamine, Hepes, and dFCS. [³⁵S]Methionine (>800 Ci/mmole, Amersham Corp.) was used at 300 μ Ci/ml and [³H]leucine (130–190 Ci/mmole, Amersham Corp.) was used at 250–350 μ Ci/ml. In all cases, cells were labeled at a density of 5–10 \times 10⁶ cells/ml for 3–5 h in a 37°C humidified atmosphere containing 7.0% CO₂. After labeling, cell suspensions were diluted in complete RPMI 1640, collected by centrifugation and cell pellets were either solubilized immediately or frozen at –80°C.

Solubilization, Immune Precipitation, and Electrophoresis. Radiolabeled cells were solubilized at a density of 2–3 \times 10⁷ cells/ml in 0.5% NP-40 (Non-Idet P-40, Particle Data, Inc., Elmhurst, IL) in PBS pH 7.2 containing the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (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 (TLCK) (50 μ g/ml) (Sigma Chemical Co., St. Louis, MO). After thorough mixing and incubation at 4°C for 45 min, the cell extracts were centrifuged at 100,000 g for 60 min at 4°C. The supernatant was collected and used as a source of Ia antigens in immunoprecipitations. Before immunoprecipitation, NP-40 lysates were pretreated once with fixed protein A-bearing *Staphylococcus aureus* Cowan strain I (SaCl) (18) to remove endogenously labeled IgG, twice with normal serum and SaCl, and finally with SaCl alone to decrease nonspecific adsorption in immunoprecipitates. Aliquots of pretreated cell lysates were incubated with antisera or monoclonal antibodies overnight at 4°C. Antigen-antibody complexes were isolated either with SaCl alone (alloantisera, 10-2.16, MKD6, Do3) or rabbit anti-rat Ig-SaCl immunoadsorbents (In-1). After 30 min at 4°C, the complexes were collected by centrifugation at 2,500 g, and washed four times with PBS containing 0.25% NP-40 (PSN).

Radiolabeled antigen was dissociated from antibody and SaCl by boiling for 2 min in SDS elution buffer (2% SDS, 62 mM Tris, pH 6.8) containing 2% 2-mercaptoethanol

² Hauptfeld, V., M. Nahm, M. Hauptfeld and D. C. Shreffler. 1983. Monoclonal antibodies to mouse MHC antigens. I. Serological characterization of 10 anti-H-2 and anti-Ia reagents. Manuscript submitted for publication.

unless otherwise indicated. Samples were analyzed by slab gel electrophoresis in a modified Laemmli-Maizel discontinuous SDS-PAGE system (18) using 10% polyacrylamide running gels and 4% polyacrylamide stacking gels. Molecular weights were estimated from the position of co-electrophoresed ^{14}C -labeled standard proteins including bovine serum albumin (mol wt 69,000) IgG (mol wt 55,000 and 25,000), ovalbumin (mol wt 46,000) and carbonic anhydrase (mol wt 30,000) purchased from New England Nuclear, Boston, MA.

Two-dimensional separations (2D-PAGE) were performed as generally described by O'Farrell (19), with some modifications (20). The first dimension separation by isoelectric focusing (IEF) was performed in 5-mm \times 130-mm cylindrical gels containing 4.0% pH 3.5–10 Ampholines (LKB Instruments, Rockville, MD). Samples were electrophoresed at 0.8 W/gel until the voltage reached 800 V, then under constant voltage (800 V) for 16 h, and then at 1,200 V for the final hour. The pH gradient was determined by measuring the pH of 1-cm slices of a blank gel run in parallel with the samples. The IEF gels were equilibrated in SDS, embedded in agarose on the top of a slab gel (3-cm 4% acrylamide stacking gel and 13-cm 11% acrylamide running gel) and electrophoresed as described previously (20).

Protease Treatments. Staphylococcal V8 protease (*S. aureus* V8 lot 13) was purchased from Miles Laboratories (Elkhart, IN). A 1 mg/ml stock solution of enzyme was prepared in SDS elution buffer and added to SDS eluates of immunoprecipitates at a final concentration of 125 $\mu\text{g}/\text{ml}$. Enzyme-treated and untreated control samples were incubated at 37°C for 2 h, then placed in a boiling water bath for 2 min to inactivate enzyme. 2-Mercaptoethanol was added to a final concentration of 2% and samples were immediately electrophoresed on 10% SDS-PAGE slab gels.

For pronase digestion, eluates of SaCl immunoprecipitates were precipitated with 3 volumes of 95% ethanol containing 1.3% potassium acetate at -20°C (21). Carrier protein was included at a final concentration of 1 mg/ml and precipitation was allowed to proceed overnight at -20°C . The 4,100 g pellets were washed three times in 95% ethanol, 1.3% potassium acetate, once in absolute ethanol, and were then dried overnight at 37°C. Ethanol precipitates were solubilized in 0.5 ml of 10 mM Tris, 1 mM CaCl_2 , pH 8. Predigested pronase (*S. griseus* protease, lot 202867; Calbiochem-Behring Corp., San Diego, CA) was added to a final concentration of 5 mg/ml. Samples were incubated in a toluene atmosphere at 50°C for 12 h, a second aliquot of pronase equivalent to the first was added, and digestion was allowed to continue for an additional 12 h. A control sample was incubated in the digestion buffer in the absence of enzyme for 24 h at 50°C. After digestion, samples were boiled for 2 min to inactivate enzyme and either used directly or stored at -80°C .

Bio-Gel P-10 Chromatography. Pronase-treated samples (0.5 ml) were applied to a Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, California) column (-400 mesh, 40-cm \times 1-cm) equilibrated in 0.1 M ammonium bicarbonate. The column was eluted with the same buffer at a flow rate of 1.5 ml/h. Fractions of 0.9 ml were collected and the radioactivity in an aliquot from each fraction was determined. Void (V_0) and included (V_i) volumes were determined for each column run by monitoring hemoglobin (V_0) and mannose (V_i), which were added to each sample before application to the column. Bio-Gel P-10 has an exclusion limit of 20,000 for globular proteins.

Alkaline Borohydride Treatment. An aliquot of the $^{35}\text{SO}_4$ -labeled fraction peak obtained by Biogel-P-10 chromatography of pronase-digested material was lyophilized to dryness, resuspended in 1.0 ml hydrolysis buffer (0.05 M NaOH, 5×10^{-5} M *N*-acetyl-glucosamine, 1 M NaBH_4) and incubated at 45°C for 16 h (22). Acetic acid was then added dropwise to neutralize the sample. Sodium ions were removed by passing the solution over a 2-ml column of activated Dowex AGWX8 (Bio-Rad Laboratories) and borate ions were removed by evaporating the Dowex column effluent three times with methanol. The dried sample was resuspended in 0.1 M ammonium bicarbonate, reconstituted with hemoglobin (2 mg) and mannose (2 mg) and rechromatographed on the Bio-Gel P-10 column.

Chondroitinase Digestion. Immunoprecipitated material from $^{35}\text{SO}_4$ -labeled B10.A spleen cells was digested with pronase and chromatographed on Bio-Gel P-10. Peak

fractions, eluting in the void volume, were pooled and lyophilized to dryness, then resuspended in 50 mM Tris-HCl, pH 8, containing 0.1 mg/ml BSA. Chondroitinase AC (Miles Laboratories) was added at a final concentration of 1 U/ml and digestion was carried out for 14 h at 37°C. Chondroitin sulfate (Sigma Chemical Co.) was digested in parallel as a positive control for the efficacy of digestion. Mock treated and enzyme-digested samples were boiled for 2 min and then rechromatographed on Bio-Gel P-10.

Paper Chromatography. Chondroitinase AC-digested samples were spotted on Whatman no. 1 paper (American Scientific Products) and analyzed by descending paper chromatography using a solvent system of 1-butanol, glacial acetic acid, and 1 N NH₄OH (2:3:1). Disaccharide standards run in adjacent lanes included 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-4-*O*-D galactose (Δ -Di-4S), 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-6-*O*-sulfo-D-galactose (Δ -Di-6S) and 2-acetamido-2-deoxy-3-*O*-(β -D-glucopyranosyluronic acid)-D-galactose (Δ -Di-OS).

Results

A Novel [³⁵S]Sulfate-Labeled Component Coprecipitated with Ia. To determine whether Ia glycoproteins are sulfated, splenocytes from B10.A mice were divided into two groups and biosynthetically labeled either with [³H]leucine or with ³⁵SO₄. After labeling, cell pellets were solubilized in 0.5% NP-40, and aliquots of the cell lysates were immunoprecipitated with several alloantibodies (Fig. 1A). H-2K^k and Ia^k glycoproteins labeled with [³H]leucine are shown in lanes 2–4. When gel profiles of immunoprecipitates prepared from [³⁵S]sulfate-labeled spleen cells were analyzed, two observations were made. First, there was no detectable labeled band in the anti-H-2K immunoprecipitate (lane 6). Second, anti-Ia immunoprecipitates from ³⁵SO₄-labeled spleen cells did not contain labeled bands in the region of the gel where Ia α and β or associated invariant (I_i) glycoproteins migrate. Instead, a labeled band of considerable molecular weight heterogeneity was seen, spanning the 46–69,000 dalton (46–69 kd) region of the gel. This material was detectable only in the immunoprecipitates prepared with an anti-Ia alloantiserum (lane 8) and with an anti-I-A^k monoclonal antibody (lane 7), and was not found in the normal serum control (lane 5). We confirmed and extended these observations in the following two experiments.

B10.BR spleen cells were labeled with [³⁵S]sulfate and immunoprecipitates from the NP-40 cell lysate were examined (Fig. 1B). The monoclonal anti-I-A antibody precipitated a band similar to that seen with B10.A splenocytes (lane 5). In addition, when a monoclonal anti-I-E antibody was used for immunoprecipitation, the same type of band was detectable (lane 6). Analysis of immunoprecipitates prepared with SaCl alone to precipitate endogenous IgG, or with an anti H-2K alloantiserum showed no detectable [³⁵S]sulfate-labeled bands (lanes 1 and 3). Control precipitates employing normal mouse serum (NMS) or an irrelevant monoclonal antibody were also negative (lanes 2 and 4). The results of a second experiment using B10.A spleen cells are shown in Fig. 1C. The 46–69-kd component was not only seen in immunoprecipitates prepared with the monoclonal anti-I-A and -I-E reagents (lanes 2 and 3), but also in an immunoprecipitate prepared with a monoclonal anti-invariant chain reagent (lane 5). Control precipitates prepared with an irrelevant monoclonal antibody or with the rabbit anti-rat Ig developing reagent (lanes 1 and 4) were negative. In other experiments (not shown), SDS gel analysis of ³⁵SO₄-labeled B10.A immunoprecipitates prepared with rabbit anti-mouse Ig or anti-H-2D^d alloantisera also do not show any

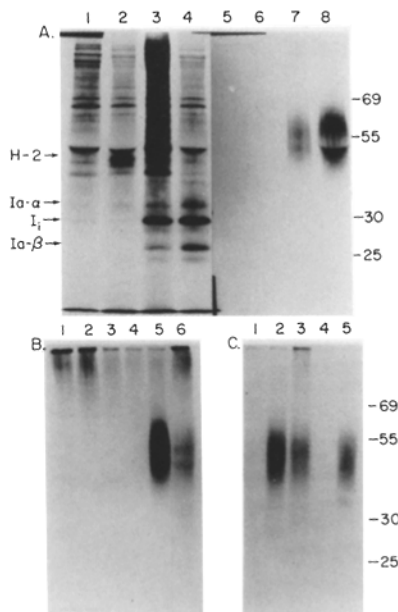


FIGURE 1. SDS-PAGE identifies a 46–69,000-dalton $^{35}\text{SO}_4$ -labeled component precipitated by anti-Ia reagents. (A) [^{35}S]Methionine-labeled (lanes 1–4) or $^{35}\text{SO}_4$ -labeled (lanes 5–8) B10.A spleen cell extracts were precipitated with normal mouse serum (lanes 1 and 5), anti-H-2K^k alloantiserum (lanes 2 and 6), anti-I-A^k monoclonal antibody (lanes 3 and 7) and anti-I^k alloantiserum (lanes 4 and 8). (B) A $^{35}\text{SO}_4$ -labeled B10.BR spleen cell extract was precipitated with protein-A bearing Staphylococci only (lane 1), normal mouse serum (lane 2), anti-H-2K^k alloantiserum (lane 3), control anti-I-A^d monoclonal antibody (lane 4), anti-I-A^k monoclonal antibody (lane 5), and anti-I-E^k monoclonal antibody (lane 6). (C) A $^{35}\text{SO}_4$ -labeled B10.A spleen cell extract was precipitated with control anti-I-A^d monoclonal antibody (lane 1), anti-I-A^k monoclonal antibody (lane 2), anti-I-E^k monoclonal antibody (lane 3), control rabbit anti-rat Ig-SaCI immunoadsorbent (lane 4), and anti-I_i monoclonal antibody plus rabbit anti-rat-Ig-SaCI (lane 5).

labeled bands.

The results of the experiments shown in Fig. 1 indicate that immunoprecipitates prepared with antibodies specific for components of the Ia molecular complex contain not only α , β and I_i chains but also an additional sulfate-bearing molecule, of marked molecular weight heterogeneity. This molecule appears to be specifically associated with Ia since it was not precipitated in association with other spleen cell proteins, such as immunoglobulin or class I MHC antigens.

To establish that the 46–69-kd molecule is expressed in mice of different H-2 haplotypes, spleen cells from H-2^b, H-2^d, and H-2^s mice were biosynthetically labeled with $^{35}\text{SO}_4$, and lysed with NP-40. Labeled molecules were immunoprecipitated with the anti-invariant chain monoclonal antibody. The results shown in Fig. 2 demonstrate that these other haplotypes do indeed express the 46–69-kd molecule.

In the preceding experiments, immunoprecipitates were analyzed by electrophoresis in the presence of reducing agents. In order to determine whether the 46–69-kd product was covalently associated with Ia glycoproteins, we compared the electrophoretic behavior of [^3H]leucine and $^{35}\text{SO}_4$ -labeled Ia precipitates in

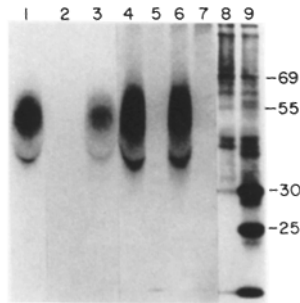


FIGURE 2. SDS-PAGE analysis of anti-Ii immunoprecipitates from mice of different H-2 haplotypes. Spleen cells from B10.A (lane 1), B10.S (lanes 2 and 3) B10.D2 (lanes 4 and 5) or C57BL/10 (lanes 6 and 7) mice were biosynthetically labeled with $^{35}\text{SO}_4$ and lysed with NP-40. Lysates were immunoprecipitated with either R α RIg alone (lanes 2, 5, and 7) or anti-I_i and R α RIg (lanes 1, 3, 4, and 6). [^3H]Leucine-labeled B10.S R α RIg (lane 8) and anti-I_i (lane 9) immunoprecipitates are shown for comparison.

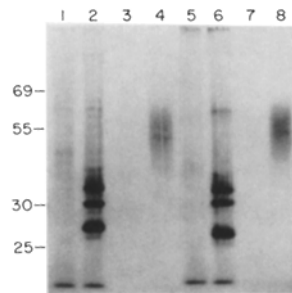


FIGURE 3. SDS-PAGE of [^3H]leucine and $^{35}\text{SO}_4$ -labeled B10.A I-A immunoprecipitates with and without 2-mercaptoethanol. Precipitates in lanes 1–4 were treated with 2-ME while precipitates in lanes 5–8 were untreated. Control precipitates (lanes 1, 3, 5, and 7) employed an irrelevant monoclonal anti-I-A^d reagent, and specific precipitates (lanes 2, 4, 6, and 8) used monoclonal anti-I-A^k antibody.

the presence or absence of 2-mercaptoethanol (Fig. 3). A comparison of reduced (lane 2) and nonreduced (lane 6) [^3H]leucine-labeled immunoprecipitates demonstrates that α , β , and I_i chains undergo only minor mobility shifts upon reduction, which is expected from cleavage of intrachain disulfide bonds. The relative mobility of the $^{35}\text{SO}_4$ -labeled component was the same in the presence (lane 4) and absence (lane 8) of 2-ME, indicating that the interaction of this molecule with α , β , and/or I_i chains is noncovalent. These results also demonstrate that the 46–69-kd product is not a disulfide-linked subunit of a larger molecular weight species.

Biochemical Characteristics of the 46–69-kd Molecule. Despite the prominence of the 46–69-kd component in precipitates from [^{35}S]sulfate-labeled spleen cells, no specific [^{35}S]methionine- or [^3H]leucine-labeled bands were detectable in the 46–69-kd region in SDS gel analysis of I_a or I_i immunoprecipitates. There are several possible explanations that could account for our inability to detect specific bands labeled with amino acid precursors by this method. If the [^{35}S]sulfate-labeled component contains protein, it may constitute a very minor percentage of total spleen cell proteins. Because no purification steps were performed before im-

munoprecipitation, such a minor species could readily be obscured by the proteins that generally contaminate immune precipitates of this type. Alternatively, the turnover time of the $^{35}\text{SO}_4$ -bearing protein could be slow relative to that of Ia and I_i glycoproteins and thus it would be poorly labeled with [^3H]-leucine or [^{35}S]methionine under the relatively short-term labeling conditions used. A third possibility is that the material may be proteoglycan in nature, and therefore it would have only a small, and thus poorly labeled, protein core to which sulfated carbohydrate chains are covalently attached. Finally, the 46–69-kd band could be a sulfated polysaccharide that is totally devoid of protein. We addressed some of these possibilities in the following experiments.

To determine whether the 49–69-kd component is labeled with [^3H]leucine or [^{35}S]methionine but is masked by other labeled proteins, we analyzed Ia and I_i immunoprecipitates by two-dimensional gel electrophoresis (2D-PAGE) (Fig. 4). Fig. 4, A and B shows 2D gels of the $^{35}\text{SO}_4$ -labeled Ia and I_i immunoprecipitates. Two major sulfate-labeled species, distinguishable by charge and apparent molecular weight, were observed in both Ia and I_i immunoprecipitates. One component was detected as a very diffuse spot, migrating between pH 4.2 and 5.0 and molecular weight 40–50 kd. The other component was less heteroge-

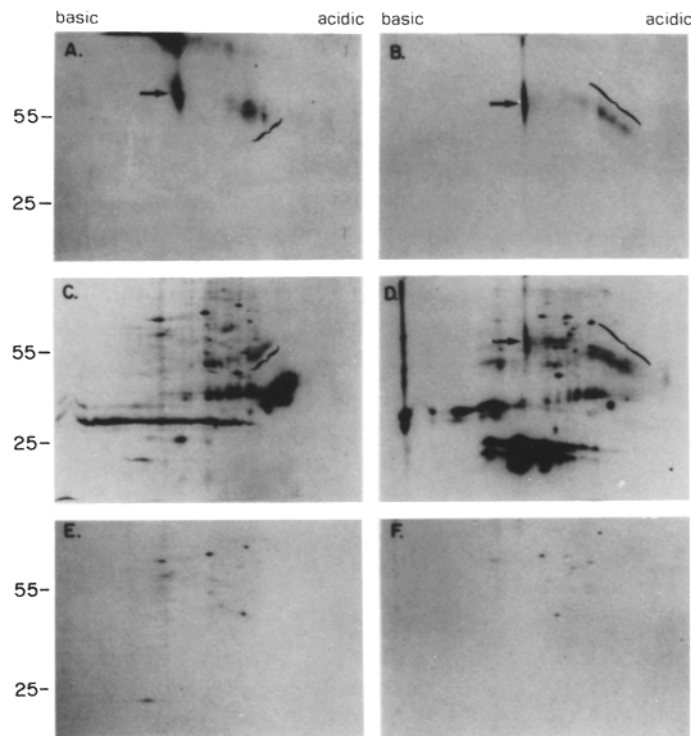


FIGURE 4. 2D-PAGE analysis of [^{35}S]methionine/[^3H]leucine and $^{35}\text{SO}_4$ -labeled I-A and I_i immunoprecipitates from B10.A spleen cells. $^{35}\text{SO}_4$ -labeled I-A (panel A) and I_i (panel B) are compared to ^{35}S -met/ ^3H -leu I-A (panel C) and I_i (panel D). Control panels (E and F) show precipitates made from ^{35}S -met/ ^3H -leu labeled material with rabbit anti-rat immunoglobulin (panel E) or with an irrelevant (anti I-A^d) monoclonal antibody (panel F). The control $^{35}\text{SO}_4$ precipitates (not shown) were negative.

neous in charge, with a pI of ~ 6.0 and was of slightly higher average molecular weight than the pI 4.2–5 species.

When these gels were compared with those from the [^3H]leucine- and [^{35}S]methionine-labeled anti I-A and I_i immunoprecipitates (Fig. 4, C and D, respectively), specific spots corresponding to the more acidic sulfate-labeled spots were detected in both I_i and I-A immunoprecipitates (brackets) upon long exposure of the gel to film, indicating that this sulfate-labeled component contains polypeptide components. The more basic species is easily detectable in the [^{35}S]methionine- and [^3H]leucine-labeled I_i immunoprecipitate (arrow), but was not detected in the [^{35}S]methionine- and [^3H]leucine-labeled I-A immunoprecipitate. We have found that visualization of the more basic species to be somewhat variable, although the reason for this is not clear at this time.

To further establish that all the $^{35}\text{SO}_4$ -labeled immunoprecipitated material contains protein, Ia and I_i immunoprecipitates from a $^{35}\text{SO}_4$ -labeled B10.A cell lysate were subjected to degradation by *S. aureus* V8 protease, which cleaves at glutamyl and aspartyl residues (23). ^{14}C -BSA and ^{14}C -bovine gamma globulin were treated in parallel as positive controls for digestion. Protease-treated and mock-digested untreated samples were then electrophoresed on 10% SDS gels (Fig. 5). Lanes 1, 3, 5, and 7 show the mock-digested ^{14}C -BSA, ^{14}C -BGG, $^{35}\text{SO}_4$ -I_i, and $^{35}\text{SO}_4$ -I-A immunoprecipitates, respectively. Lanes 2, 4, 6, and 8 show the enzyme-treated samples. Both the anti-I_i- and anti-I-A-precipitated [^{35}S]sulfate-labeled products were reduced in apparent molecular weight by the V8 protease treatment, indicating that both $^{35}\text{SO}_4$ -labeled components seen on the 2D gels contain protein. The material remaining after V8 digestion continued to have heterogeneous mobility, but was all below 40 kd in apparent molecular weight.

We wished to gain further information on the chemical nature of the 46–69-kd sulfated component of Ia and I_i precipitates. In particular, we were interested in the manner in which sulfate was linked to this molecule and to what class of macromolecules it belonged. Sulfate may be attached to biological molecules in several different ways. Proteoglycans contain sulfate groups O- or N-linked to sugar residues of large glycosaminoglycan side chains, which in turn are covalently attached to the core protein (reviewed in reference 24). Recently, sulfate

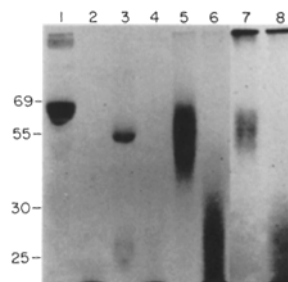


FIGURE 5. Treatment of the $^{35}\text{SO}_4$ -labeled component with Staphylococcal V8 protease. ^{14}C -Labeled BSA without V8 treatment (lane 1) is reduced to low molecular weight species after digestion (lane 2). Similarly ^{14}C -labeled IgG without V8 treatment (lane 3) is reduced to low molecular weight species after digestion (lane 4). The $^{35}\text{SO}_4$ -labeled components precipitated by anti-I_i (lane 5) or anti-Ia (lane 7) are reduced in apparent molecular weight by V8 protease (lanes 6 and 8, respectively) from 46–69,000 to <40,000.

has been identified on sugar residues of O- and N-linked oligosaccharides on mammalian glycoproteins (9–12) and has also been found linked directly to amino acid residues of proteins (13). Several features of the 46–69-kd molecule, including its heterogeneity in molecular weight by SDS-PAGE and its poor incorporation of amino acid precursors, suggested that it might be proteoglycan in nature. We investigated this possibility in the following experiment. An NP-40 lysate of [^{35}S]sulfate-labeled B10.A spleen cells was immunoprecipitated with the monoclonal anti-invariant antibody and the immunoprecipitated material was subjected to exhaustive pronase treatment. We then analyzed the pronase digest by gel filtration on Bio-Gel P-10 to determine whether sulfate was associated with the released amino acids or small peptides, or remained associated with a larger pronase-resistant moiety (Fig. 6). A single radioactive peak was recovered, eluting in the void volume of the column. This result was suggestive evidence that the sulfate-bearing moiety was a relatively large macromolecule, since amino acids and conventional glycopeptides bearing O- or N-linked oligosaccharide groups would be found in the included volume of the column. However, we did consider the possibility that the sulfated material was pronase resistant because it contained clusters of serine or threonine residues to which small O-linked sulfated carbohydrate groups were attached. Such structures have been reported to be pronase resistant (22, 25). To address this question, we treated an aliquot of the pronase-resistant P-10 pool with alkaline borohydride under conditions that would release O-linked carbohydrate groups by β -elimination. When the treated sample was rechromatographed on the Bio-Gel P-10

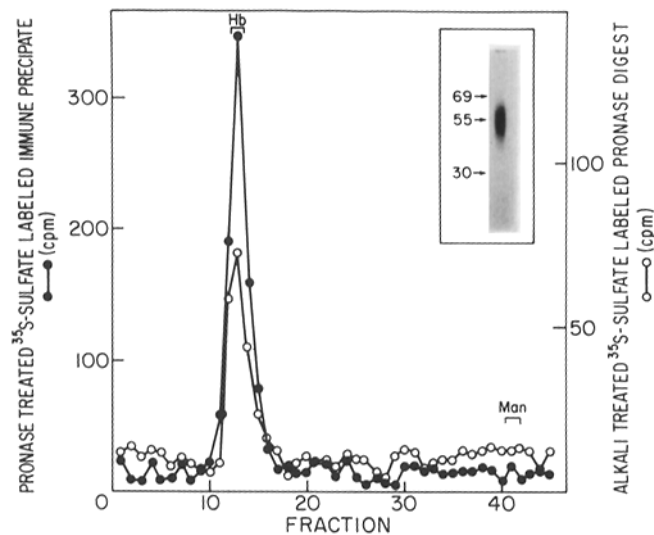


FIGURE 6. Bio-Gel P-10 chromatography of pronase and alkaline borohydride treated $^{35}\text{SO}_4$ -labeled component. $^{35}\text{SO}_4$ -labeled B10.A spleen cell lysate precipitated with anti-I₁ was exhaustively digested with pronase and then analyzed on a sizing column of Bio-Gel P-10 (●). Fractions 11–15 of pronase treated, P-10 fractionated sample were pooled and, an aliquot was subjected to mild base hydrolysis, then re-chromatographed on the Bio-Gel P-10 column (○). The elution positions of hemoglobin (Hb) and free mannose (Man) are indicated. *Inset* SDS-gel profile of the anti-I₁ immunoprecipitate that was pronase digested.

column, its elution position was unchanged (Fig. 6). This result rules out the possibility that the $^{35}\text{SO}_4$ was attached to small O-linked oligosaccharides, and indicates that the sulfate residues are borne on a large glycoconjugate, typical of proteoglycans.

To establish that the 46–69-kdalton molecule was indeed a proteoglycan, its sensitivity to enzymes that are specific for the carbohydrate units of proteoglycans was tested. The glycosaminoglycan components of proteoglycans consist of long repeating units of disaccharides consisting of a hexosamine and, with the exception of keratan sulfate, a uronic acid. Initial experiments (not shown) indicated that the $^{35}\text{SO}_4$ -bearing moiety of the Ia-associated product was degraded by the enzyme chondroitinase ABC, which will degrade both dermatan sulfate and chondroitin sulfate to 4,5 unsaturated disaccharides (26). To determine which class of glycosaminoglycans the molecule belong to, anti-I-A and anti-Ii immunoprecipitated material from $^{35}\text{SO}_4$ -labeled B10.A spleen cells was subjected to degradation by chondroitinase AC, which is specific for chondroitin sulfate. Fig. 7 shows the results of these experiments. All of the sulfate-bearing species associated with Ia were sensitive to chondroitinase AC, indicating that the 46–69-kdalton macromolecule is a proteoglycan with chondroitin sulfate glycosaminoglycan side chains. Paper chromatography of the digestion products (Fig. 7, panel C) showed that the radiolabeled material migrated at the position of the 6-sulfated reference disaccharide, indicating that virtually all of the sulfation in this molecule occurs at the 6-position of *N*-acetylgalactosamine.

Discussion

The major histocompatibility complex (MHC) is critical in expression of the immune response to a variety of antigens. In particular, evidence accumulated over the past 10 years indicates that genes mapping to the I region of the MHC play a central role in dictating the magnitude of the response to a given antigen, and govern the interaction of the various cell types within the immune system (27–30). Because of this, there has been much interest in elucidating the structure of products encoded within this subregion. Early studies of immunoprecipitated Ia molecules indicated that both the I-A and I-E subregion products consist of genetically polymorphic glycoprotein heterodimers composed of a 33–35-kdalton α chain, and a 25–27-kdalton β chain (18). Later studies by Jones et al. (31) documented that a third protein, termed invariant chain because of its non-polymorphic nature, was also present in anti-Ia immunoprecipitates. Although invariant chain associates specifically with Ia antigens, recent data indicate that the gene controlling this protein lies outside the major histocompatibility complex (32, 33).

In this paper, we report the identification of another component in the oligomeric Ia antigen complex—a heterogeneous sulfated protein-containing molecule migrating with an apparent molecular weight of 46–69-kdaltons by SDS-PAGE. This product could be immunoprecipitated using antibodies specific for I-A, I-E, and I_i glycoproteins, but it was not detectable in control immunoprecipitates, nor in H-2K or immunoglobulin precipitates. We have also found this molecule in other H-2 haplotypes and thus it is not peculiar to I^k mice. The 46–69-kdalton component is not disulfide-linked to Ia, since its apparent molec-

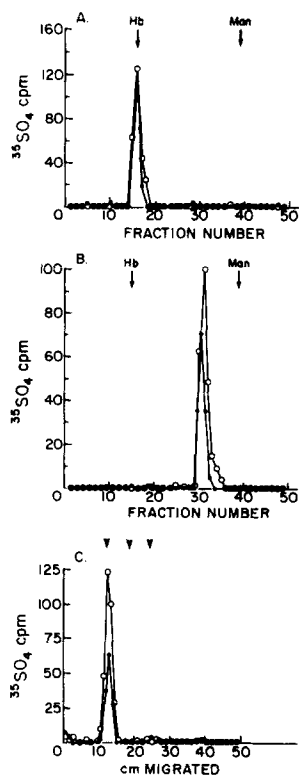


FIGURE 7. Chondroitinase AC digestion of the Ia associated sulfated product. $^{35}\text{SO}_4$ -labeled B10.A spleen cell lysate was immunoprecipitated with an anti-I-A^k (●) or anti-I-i (○) monoclonal antibody. Immunoprecipitates were digested with pronase, fractionated on Bio-Gel P-10 and material eluting in the void volume was pooled and lyophilized to dryness. Samples were resuspended in 50 mM Tris HCl, pH 8 containing 0.1 mg/ml BSA. Aliquots were incubated in the presence or absence of chondroitinase AC then rechromatographed on Bio-Gel P-10. The chromatographic profile of the mock-treated samples, (panel A) and the chondroitinase-digested samples (panel B) are shown. An aliquot of the chondroitinase digested material was then analyzed by paper chromatography (panel C). Arrowheads indicate the relative migration of disaccharide standards run in adjacent lanes (from left to right: Δ -Di-6S, Δ -Di-4S, and Δ -Di-OS). Unlabeled chondroitin sulfate, digested in parallel with the samples yielded both 4- and 6-sulfated disaccharides.

ular weight on SDS-PAGE is the same in the presence or absence of reducing agents. The cellular expression of the sulfated product is similar to that observed for conventional Ia antigens. It is present in Ia precipitates of splenic B cells and continuous B cell lines but is not detectable in Ia precipitates of splenic T cells or thymocytes (manuscript in preparation).

Preliminary biochemical studies of the 46–69-kdalton molecule suggested to us that it might be a proteoglycan. These studies indicated that this molecule was extremely heterogeneous, both in charge and apparent molecular weight, a common feature in proteoglycans (33). We found the molecule to be sensitive to proteases but poorly labeled with methionine and leucine under short-term labeling conditions. When pronase-digested or alkali-treated material was examined by gel filtration on Bio-Gel P-10, a single peak of radioactivity was

recovered, eluting in the void volume, thus excluding the possibility that the $^{35}\text{SO}_4$ is associated with low molecular weight material such as single amino acids, small peptides, or glycopeptides that bear oligosaccharides typical of conventional glycoproteins. The results of experiments testing the susceptibility of this molecule to enzymes specific for proteoglycans confirmed our hypothesis that it was a proteoglycan and demonstrated that this sulfated, Ia-associated product contains chondroitin 6-sulfate.

An important point raised by these studies is whether the sulfate-labeled product that we detect in our immunoprecipitates is reacting directly with the antibodies used or is precipitated only by virtue of its association with Ia or I_i. This type of question is difficult to address with an oligomeric complex such as Ia. The most direct method would be to separate individual components in the complex and test for reactivity of the antibodies with the isolated molecules. A negative result in this experiment would be difficult to interpret, however. Even with the conventional Ia heterodimer, it has often been difficult to assign serological specificities to α or β chains, most commonly because immune reactivity is lost upon denaturation and dissociation of the dimer. Several points in our experiments do bear on this question, however. First, we detected this molecule not only in immunoprecipitates prepared with alloantiserum but also in those prepared with monoclonal antibodies. This rules out the possibility that the sulfated product bears MHC-encoded determinants distinct from conventional Ia but reactive with antibodies other than anti-Ia that are contained within a complex alloantiserum. Secondly, the monoclonal antibodies we have used are clearly reactive against different glycoproteins. Do3 detects Ia.7, a public specificity on I-E molecules. 10-2.16 recognizes Ia.17, present on I-A^{k,f,r,s} molecules. In-1 reacts with the nonpolymorphic invariant proteins that are not MHC-encoded. Thus, although it is possible that the 46–69-kdalton material reacts directly with one of these antibodies, we feel it is unlikely that it reacts directly with all three.

Assuming that this product is immunoprecipitated because of its noncovalent interaction with the Ia complex, it is important to determine if it is primarily associated with only one of the glycoproteins, i.e., α , β , or I_i, and when and where this association occurs. Of particular interest is what role this molecule might play in the biosynthesis of Ia, in the formation of the α , β , I_i oligomeric complex and in the expression of Ia antigens at the plasma membrane. Experiments designed to address these issues are currently underway in our laboratory.

Summary

In this report, we describe a previously unidentified component in the murine Ia antigen complex. SDS-PAGE analysis of anti-Ia immunoprecipitates prepared from spleen cells biosynthetically labeled with ^{35}S -sulfate showed no detectable incorporation of $^{35}\text{SO}_4$ into α , β , or I_i chains but did not reveal the presence of a novel sulfate-bearing molecule of considerable molecular weight heterogeneity (46–69-kdaltons). The 46–69-kdalton molecule could be precipitated with monoclonal antibodies specific for I-A, I-E, and I_i glycoproteins but was not seen in control precipitates, nor in association with IgG or class I MHC molecules. Preliminary biochemical characterization indicated that the 46–69-kdalton prod-

uct is extremely polydisperse, both in charge and apparent molecular weight, is sensitive to proteases, and bears the sulfate moiety on a large pronase-resistant structure. These results suggested this component might be a proteoglycan. Definitive identification of this component as a proteoglycan was accomplished by selective enzymatic degradation experiments which showed that the sulfate-bearing component of the 46–69-kdalton molecule is chondroitin 6-sulfate.

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