LYMPHOCYTE MEMBRANE IgG AND SECRETED IgG ARE STRUCTURALLY AND ALLOTYPICALLY DISTINCT*

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The monomeric IgM and IgD molecules on the cell surface of B lymphocytes are generally thought to be integral membrane antigen receptors (1–3). These molecules are believed to be involved in triggering B cell activation (4–6) and, as such, might be expected to be structurally different from their secreted homologues (at least in the case of IgM). Currently there are conflicting reports as to the chemical nature of the difference between membrane and secreted IgM (7–10), but there appears to be general agreement that the two molecules are structurally different (11–13).

Subpopulations of B cells also have been shown to express surface IgG (1, 14–16). Because IgG may be the only antigen receptor on the most mature memory B cell subpopulation (17), membrane IgG also might be expected to be structurally different from its secreted homologue.

In this communication, we show that there are distinct membrane and secreted IgG_{2a} molecules, distinguished by biochemical and serological criteria. The membrane IgG_{2a} studied here is derived from a cloned B-lymphoma cell line, 2PK3 (18, 19), which produces both membrane and secreted IgG_{2a} immunoglobulin. The heavy chain constant region of the secreted immunoglobulin is the gene product of the *a* allele of the *Igh-1* locus (20). We demonstrate two major differences between membrane and secreted molecules: (*a*) membrane and secreted IgG_{2a} heavy chains have different apparent molecular weight (Mr)[1] and net charge as shown with two-dimensional polyacrylamide gel electrophoresis; and (*b*) membrane IgG_{2a} is missing an allotypic determinant present on secreted IgG_{2a} as detected with a hybridoma-derived anti-Igh-1a antibody.

The significance of these observations for immunoglobulin gene organization and control of expression of membrane and secreted heavy chain homologues is discussed. We mention further the potential biological significance of the structural differences between membrane and secreted heavy chains for the regulation of immunoglobulin synthesis.

Materials and Methods

Tumor Cells. The 2PK3 tumor cell line was obtained from Dr. Noel Warner, University of New Mexico, Albuquerque, N. M. Before any analyses of these tumor cells were undertaken,

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[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; FCS, fetal calf serum; Mr, apparent molecular weight(s); mRNA, messenger RNA(s); NP-40, Nonidet P-40; PBS, mouse-tonicity phosphate-buffered saline; SaC, *Staphylococcus aureus* Cowan I strain; SDS, sodium dodecyl sulfate; SV40, simian virus 40; T, transformation; TSTA, tumor-specific transplantation antigen.

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the cell line was cloned with the fluorescence-activated cell sorter (FACS, Becton, Dickinson FACS Systems, Mountain View, Calif.). Cells were routinely maintained in stationary flasks at 37°C in RPMI-1640 medium (Irvine Scientific, Santa Ana, Calif.) that contained 15% fetal calf serum (FCS), 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin in a humidified 7% CO_2 in air atmosphere.

Biosynthetic Radiolabeling. Immunoglobulin derived from 2PK3 cells was radiolabeled biosynthetically by incubating the cells in lysine- and arginine-free RPMI-1640 medium that contained 10 μCi/ml of both [^{14}C]lysine and [^{14}C]arginine (New England Nuclear, Boston, Mass.) and 10% FCS. Cells also were labeled biosynthetically with 20 μCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, Ill.) in methionine-free medium. Initial cell densities for isotope incorporation were 1–2 × 10^6 cells/ml and incorporation times were 16–24 h at 37°C in a humidified 7% CO_2 in air atmosphere. Supernates from biosynthetically labeled cultures were used for analysis of secreted IgG. Cell extracts were used for analysis of cellular IgG. Extracts were prepared by washing 2–4 × 10^6 radiolabeled cells twice in serum-free medium and solubilizing the cell pellet in 1 ml of 0.5% Nonidet P-40 (NP-40; Particle Data, Inc., Elmhurst, Ill.) in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM EDTA, 0.02% sodium azide, and 50 mM phenylmethylsulphonylfluoride, pH 8.0. Solubilizatiion was for 20 min on ice with occasional vortexing, followed by centrifugation for 10 min at 12,000 *g*. Cell extracts and supernates were stored at −70°C.

Cell Surface Radioiodination. Cell suspensions with viabilities >95% were surface-labeled by the lactoperoxidase method. 2 mCi of Na[^{125}I] (Amersham Corp.) were used to label 5–10 × 10^7 cells in 100–200 μl of mouse-tonicity phosphate-buffered saline (PBS), pH 7.3 (7.7 g NaCl, 0.54 g KH_2PO_4, and 2.27 g Na_2HPO_4 per liter). 10 μg of lactoperoxidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in 50 μl of PBS were added to the cell suspension followed by four 10-μl pulses of H_2O_2 at increasing concentrations (0.001%, 0.003%, 0.01%, and 0.03% in PBS) at 2-min intervals. 2 min after the last pulse, the cells were washed twice in 5 ml of PBS and solubilized as described.

Immunoprecipitation. Heat-killed and formalin-fixed *Staphylococcus aureus* Cowan I strain (SaC) organisms (The Enzyme Center, Boston, Mass.) were used for direct and indirect immunoprecipitations (21) of cell extracts and culture supernates. Because the protein A coat protein of *S. aureus* binds mouse IgG_{2a} (22), SaC was used to directly precipitate 2PK3 IgG_{2a} immunoglobulin. For indirect immunoprecipitation with hybridoma antibodies (see below), it was necessary to precoat the SaC with these antibodies, saturating all protein-A-binding sites. All the antibodies used were IgG_{2a} proteins. 1 mg of purified antibody incubated with 50 μl of a 10% suspension of SaC for 20 min on ice was sufficient for saturation. Excess antibody was removed by washing the coated SaC three times in a high-salt-NP-40 wash buffer (0.5% NP-40, 50 mM Tris-HCl, 0.45 M NaCl, and 0.02% NaN_3, pH 8.3). To minimize nonspecific binding of labeled proteins to SaC, extracts and supernates were precleared with GPC-8 (Igh-1a) myeloma protein-saturated SaC. 1 ml of cell extract (representing 2 × 10^6 cells) or 2 ml of culture supernate was precleared with 20–50 μl of GPC-8-coated SaC, followed by centrifugation for 10 min at 1,500 *g*. All immunoprecipitates were washed with high-salt-NP-40 wash buffer (see above) that contained 2 mM lysine, 2 mM arginine, 2 mM methionine, and 5 mM KI before gel analysis. Labeled proteins were eluted from the SaC with isoelectrofocusing sample buffer. (23).

Polyacrylamide Gel Electrophoresis. The two-dimensional polyacrylamide gel system utilizing nonequilibrium pH-gradient electrophoresis for the first dimension and 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis for the second dimension has been described previously (24–26). One-dimensional SDS-polyacrylamide slab gels are identical to the second dimension of the two-dimensional system. Autoradiography of gels was from 1–3 d. Gels with ^{14}C- and ^{35}S-labeled proteins were exposed to Kodak No-screen film (Eastman Kodak Co., Rochester, N. Y.) at room temperature; and gels with ^{125}I-labeled proteins were exposed to Kodak X-omat R film at −70°C with DuPoint Cronex Lightning-Plus intensifying screens (DuPont Instruments, Wilmington, Del.).

Inhibition of Glycosylation. Tunicamycin blocks the formation of the dolichol pyrophosphate intermediate that mediates core oligosaccharide addition to specific asparagine residues of nascent polypeptide chains (27, 28). Because IgG heavy chains have a single *N*-acetylglucosaminyl-asparagine-linked oligosaccharide (29), glycosylation of IgG_{2a} heavy chains can be inhibited effectively with tunicamycin (30). Inhibition of glycosylation was with 5 μg/ml of

TABLE I

*Anti-Allotype Hybridoma Antibodies**

Anti-Igh-1a		Anti-Igh-4a	
Name	Lab designation	Name	Lab designation
Ig(1a)8.3‡	20-8.3	Ig(4a)10.9‡	20-9.10
Ig(1a)9.8‡	20-6.B8		
Ig(1a)14.4	21-74.4		
Ig(1a)15.3	21-66.3		
Ig(1a/3a)16.3	21-48.3		
Ig(1a)17.2	20-19.2		

* These antibodies were produced by hybridoma cell lines generated by somatic cell hybridization of NS-1 myeloma cells with SJL spleen cells immunized with SJA/9 anti-pertussis antibodies ([34]; V. T. Oi and L. A. Herzenberg. Manuscript in preparation.). All of these antibodies are IgG_{2a} immunoglobulins and have HL chain composition (34, 35).

‡ These antibodies have been described previously (34).

tunicamycin (obtained from Dr. Carol Sibley, University of Washington, Seattle, Wash.) present during biosynthetic labeling of 2PK3 cells with [^{35}S]methionine as described above.

Removal of N-Glycosidic-linked Oligosaccharide Chains. Although the complex *N*-glycosidic-linked oligosaccharide found on already secreted IgG_{2a} heavy chains is resistant to hydrolysis by endoglycosidase H (31, 32), the di-*N*-acetylchitobiose linkage of high mannose core oligosaccharides found on newly synthesized IgG heavy chains is susceptible to cleavage (33). Endoglycosidase H hydrolysis was carried out by incubating 0.01 U of enzyme (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) in 50 μl of PBS with the SaC precipitate of biosynthetically labeled 2PK3 cells for 1 h at room temperature. Hydrolysis was terminated by washing the SaC pellet once in high-salt-NP-40 wash buffer.

Hybridoma Antibodies. Monoclonal hybridoma anti-Igh-1a antibodies were generated and prepared as described previously (26, 34, 35). The seven antibodies used in this study are listed in Table I.

Radioiodinated Igh-1a Immunoglobulin Fragments. Preparations of Fab and Fc fragments of Igh-1a proteins by papain (Worthington Biochemical Corp., Freehold, N. J.) digestion, and $(Fab')_2$ and $(Fabc)_2$ fragments by limited proteolysis with *S. aureus* protease V8 (Miles Laboratories, Inc., Miles Research Products) was as described previously (34). Fig. 5 is a schematic diagram of the heavy chain domain composition of these fragments. These fragments were labeled with Na[^{125}I] using 1,3,4,6-tetrachloro-3α,6α-diphenylglyoluril (Iodogen, Pierce Chemical Co., Rockford, Ill.) as described (34, 36). Immunoprecipitation of labeled fragments with hybridoma antibodies was carried out using a solid-phase plate-binding procedure. Hybridoma antibodies (30 μl) were coated onto the well bottoms of flexible plastic 96-well plates (Cooke Laboratory Products, Division of Dynatech Laboratories, Alexandria, Va.) at 50 μg/ml in 50 mM Tris-HCl, 0.15 M NaCl, pH 8.1, that contained 50 μg/ml bovine serum albumin (BSA) for 1 h at room temperature. Excess reagent was removed and the free protein-binding sites in the wells were saturated by washing the plate with 1% BSA in PBS. Radioiodinated fragments in 30 μl were then added to the solid-phase antibodies for 1 h at room temperature. Excess label was removed by washing again with 1% BSA in PBS. Labeled fragments specifically bound to the plates were eluted with SDS-sample buffer for gel analysis (23).

Results

Membrane 2PK3 Immunoglobulin Is Larger and More Acidic Than Its Secreted Homologue. The immunoglobulin heavy chain precipitated from surface-iodinated 2PK3 cells with SaC or with SaC coated with hybridoma antibody Ig(1a)14.4 is larger and more acidic than the biosynthetically labeled heavy chain secreted into the

culture supernate of 2PK3 cells (Fig. 1). Fig. 1 A shows the heavy and light chains of secreted IgG precipitated from culture supernate of 2PK3 cells grown under biosynthetic labeling conditions. The line of related spots obtained from this monoclonally derived immunoglobulin heavy chain probably result from the deamination of the polypeptide chain. Similar modification of the 2PK3 light chain is less apparent.

Fig. 1 B shows the membrane heavy and light chains precipitated from surface-iodinated 2PK3 cells. The major heavy chain spots have a higher Mr and are more acidic than the secreted heavy chain. A trace of material is seen where the secreted IgG is found in Fig. 1 A. This may represent IgG just being secreted or precursor IgG from dead cells present in the cell preparation used for radioiodination. The membrane heavy chain has an Mr of ~65,000, whereas the secreted chain has an Mr of ~55,000. Both Fig. 1 A and B show identical light chain spots.

Fig. 1 C shows that both immunoglobulin molecules described above are precipitated from extracts of biosynthetically labeled 2PK3 cells. The higher molecular weight chain is in the identical position as the surface-iodinated membrane heavy chain. The lower molecular weight heavy chain, which migrated to the same position as the secreted IgG found in culture supernate, probably represents the cellular precursor to secreted IgG.

The Apparent Molecular Weight Difference between Membrane and Secreted IgG Is Not a Result of Different N-Glycosidic-linked Oligosaccharide Chains. Studies with tunicamycin and endoglycosidase H show that the difference between membrane and secreted 2PK3 IgG is not a result of different asparagine-linked glycosylation of a single heavy chain precursor. Tunicamycin selectively inhibits asparagine-linked IgG heavy chain glycosylation (30). Fig. 2 shows that after tunicamycin treatment, both membrane and secretory heavy chain from labeled 2PK3 extracts have lower Mr (panel B). In Fig. 2 and subsequent figures, only the heavy chain regions of the two-dimensional gels are shown. Blocking the addition of asparagine-linked oligosaccharide chains to 2PK3 immunoglobulin heavy chains does not affect the presence of the two biosynthetic molecules in 2PK3 cell extracts. Therefore, the Mr and net electrophoretic charge difference between membrane and secretory IgG is not a result of different *N*-glycosidic-linked oligosaccharides.

The reduction in Mr of both membrane and secreted molecules after tunicamycin treatment is about 1,000–2,000. It is interesting to note here that the preformed oligosaccharide core transferred from the dolichol pyrophosphate intermediate has an Mr of ~2,000 (28). It also should be noted, in agreement with other studies (30), that the nonglycosylated, intracellular secretory IgG made in the presence of tunicamycin is secreted as such into the culture supernate (Fig. 2 D).

Two IgG_{2a} heavy chains, corresponding to the membrane and secreted molecules, also are seen after endoglycosidase H was used to remove the high mannose *N*-glycosidic-linked oligosaccharide core from internal IgG_{2a} heavy chain precursors. The oligosaccharide chain of only some of the cellular 2PK3 heavy chains are susceptible to hydrolysis (Fig. 3). Both secretory and membrane forms are equally affected, indicating that some of both IgG_{2a} heavy chains have unprocessed oligosaccharide chains. This is seen as the new lower molecular weight spots in Fig. 3 B (compare with Fig. 3 A), which also are comparable to the nonglycosylated molecules seen after tunicamycin treatment (Fig. 2). As expected, no effect was seen with endoglycosidase H treatment of already secreted 2PK3 IgG (Fig. 3 C and D).

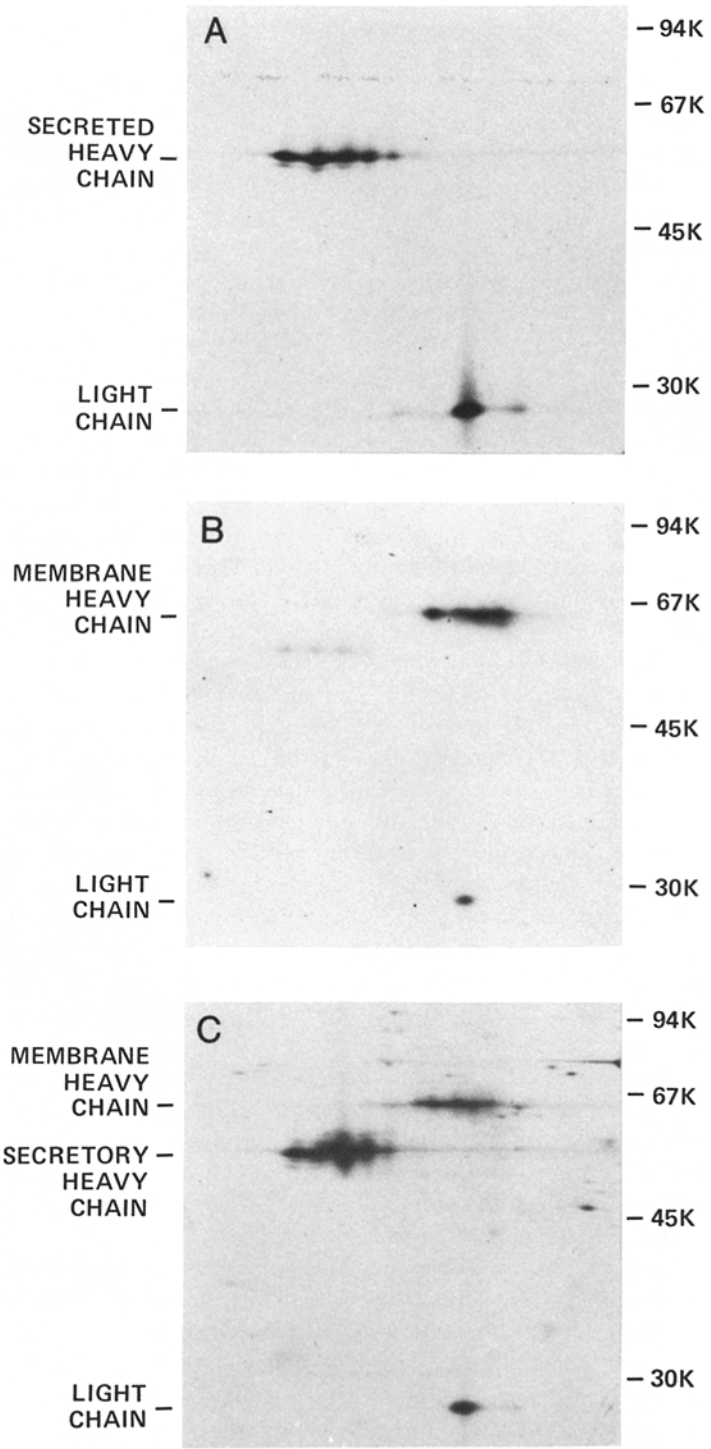

FIG. 1. Autoradiographs of two-dimensional polyacrylamide gels of secreted and membrane 2PK3 immunoglobulins. (A) Secreted IgG_{2a} precipitated from culture supernate of ^{14}C-labeled 2PK3 cells with SaC coated with hybridoma antibody Ig(1a)14.4. (B) Membrane IgG_{2a} precipitated from ^{125}I-surface-labeled 2PK3 cell extract with SaC coated with the same hybridoma antibody. (C) Cellular IgG_{2a} precipitated from ^{14}C-labeled 2PK3 cell extract with Ig(1a)14.4-coated SaC. All autoradiographic exposures were for 1 d.

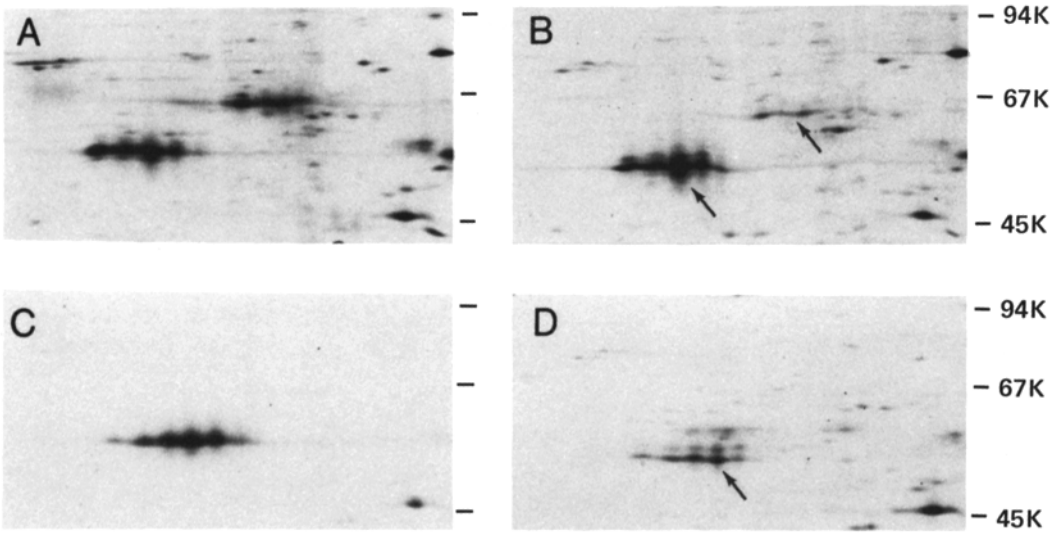

FIG. 2. Effects of tunicamycin on membrane and secreted IgG_{2a} heavy chains. (A) SaC precipitate of cell extract from [^{35}S]methionine-labeled 2PK3 cells. (B) SaC precipitate of cell extract from 2PK3 cells labeled with [^{35}S]methionine in the presence of tunicamycin. (C) SaC precipitate of culture supernate from [^{35}S]methionine-labeled 2PK3 cells. (D) SaC precipitate of culture supernate from 2PK3 cells labeled with [^{35}S]methionine in the presence of tunicamycin. In (B), arrows point to the nonglycosylated membrane and secretory heavy chains. In (D), the arrow points to the nonglycosylated secreted IgG_{2a} heavy chain. Molecular weight standards (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), indicated on the right side of each autoradiograph, were included in each gel and visualized by protein staining. The 67,000 mol wt (BSA) marker has a similar charge to the membrane IgG_{2a} heavy chain, providing an excellent two-dimensional marker for visualizing the decrease in Mr of the nonglycosylated IgG_{2a} heavy chain. All autoradiographic exposures were for 1 d. In this and subsequent figures, only the heavy-chain region of the two-dimensional gel are shown.

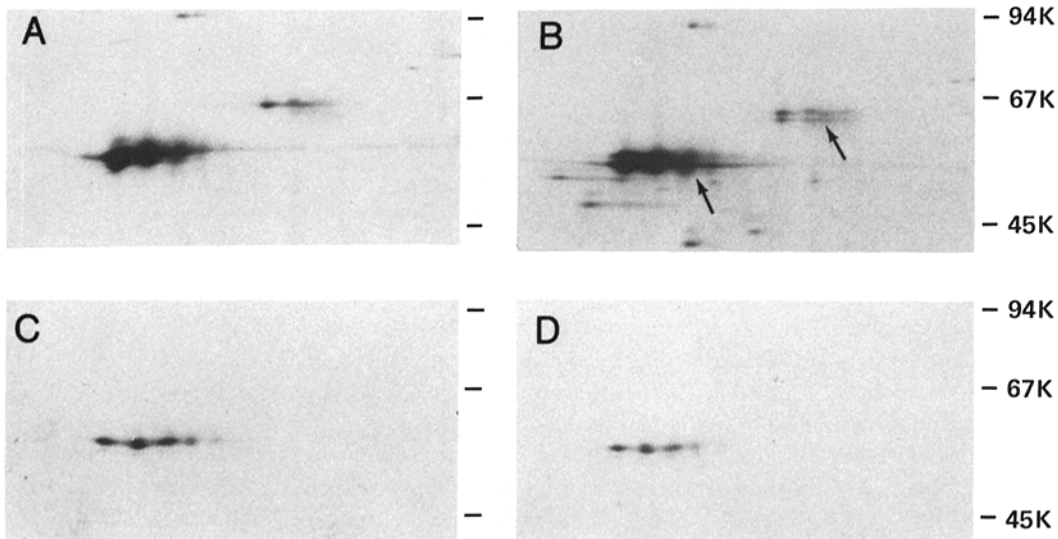

FIG. 3. Effects of hydrolysis with endoglycosidase H on membrane and secreted IgG_{2a} heavy chains. (A) SaC precipitate of secretory and membrane heavy chains from cell extracts of [^{35}S]-methionine-labeled 2PK3 cells. (B) Endoglycosidase-H-treated SaC precipitate of membrane and secretory heavy chains from extracts of [^{35}S]methionine-labeled 2PK3 cells. (C) SaC precipitate of secreted heavy chains from culture supernate of [^{35}S]methionine-labeled 2PK3 cells. (D) Endoglycosidase-H-treated SaC precipitate of secreted heavy chains from culture supernate of [^{35}S]methionine-labeled 2PK3 cells. All autoradiographic exposures were for 1 d.

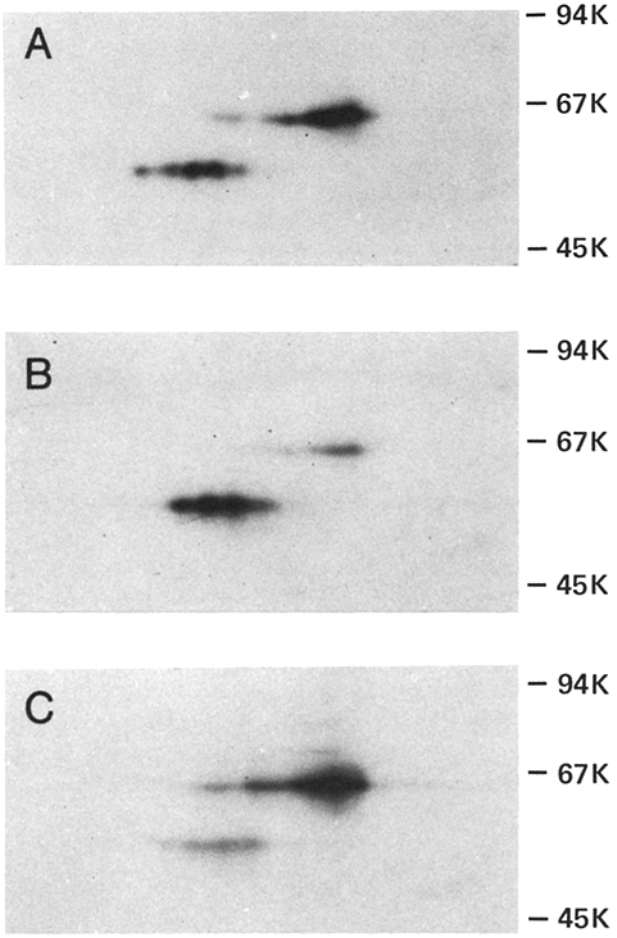

FIG. 4. Immunoprecipitation of ^{125}I-surface-labeled 2PK3 IgG_{2a} heavy chains. (A) Precipitation with Ig(1a)14.4-coated SaC (1-d autoradiographic exposure with an intensifying screen). (B) Precipitation with Ig(1a)15.3-coated SaC (3-d autoradiographic exposure with an intensifying screen). (C) Precipitation with uncoated SaC (1-d autoradiographic exposure with an intensifying screen).

Membrane IgG_{2a} Lacks an Allotypic Determinant Present on Secreted IgG_{2a}. Six different hybridoma anti-Igh-1a antibodies precipitated secreted 2PK3 IgG_{2a}, however, only five of these antibodies precipitated the membrane molecule. The sixth, Ig(1a)15.3, reacted poorly, if at all with the membrane molecule although its precipitation of secreted molecule was about equal to the other antibodies. Fig. 4 shows the gel pattern of the surface-iodinated 2PK3 cell extracts precipitated with Ig(1a)14.4 (Fig. 4A), which is typical of the five antibodies that are reactive with both IgG molecules and with Ig(1a)15.3 (Fig. 4B). The contaminating secretory-type IgG in the surface-iodinated extract clearly is evident in both precipitates. The Ig(1a)15.3 precipitate presented here shows a trace of membrane molecule; however, in other experiments, the membrane heavy chain is totally absent. It is important to note here that the autoradiograph of the Ig(1a)15.3 precipitate shown in Fig. 4B is a 3-d exposure, whereas Fig. 4A is an autoradiograph exposed for 1 d. This experimental variation

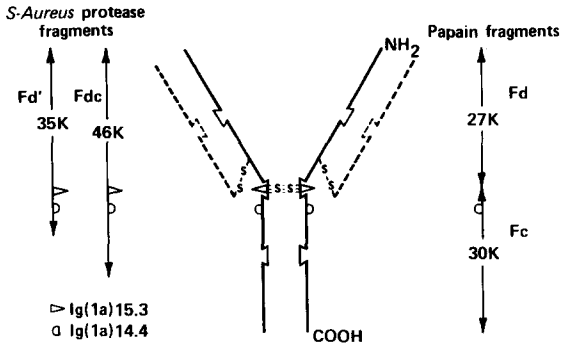

FIG. 5. Schematic diagram of enzymatic cleavage sites and location of allotypic determinants on secreted IgG_{2a} (Igh-1a). Fd and Fc are the heavy chain components of the papain-derived Fab and Fc dimer fragments. Fd consists of the V_H and C_H1 domains, and Fc consists of most of the C_H2 and all of the C_H3 domains. The hinge region and probably some of the C_H2 domain are lost with papain digestion. Fd' and Fdc are the heavy-chain components of the *S. aureus*-protease-V8-derived $(Fab')_2$ and $(Fabc)_2$ fragments. Fd' consists of the V_H, C_H1, hinge, and some of the C_H2 domains. Fdc consists of V_H, C_H1, hinge, C_H2, and probably some of the C_H3 domains. The *S. aureus* protease fragments described here are larger than was reported previously (34). This appears to be a result of the batch of enzyme used. The location of the allotypic determinants on GPC-8 myeloma protein recognized by hybridoma antibodies Ia(1a)15.3 and Ig(1a)14.4, based on results presented in Fig. 6, are shown. These results must be interpreted in view of results obtained with another Igh-1a protein presented in the text.

may reflect how efficiently the SaC is coated with hybridoma antibody; free protein-A sites on the SaC would bind the membrane form. Fig. 4C shows the precipitate using uncoated SaC.

The Location of the Missing Membrane Allotypic Determinant on Secreted IgG_{2a} Molecules. The location of the determinant recognized by each hybridoma antibody was determined by testing the reactivity of these antibodies with GPC-8 (Igh-1a) myeloma protein fragments (Fig. 5). Both Ig(1a)16.3 and Ig(1a)14.4 antibodies precipitate the Fc, the $(Fab')_2$, and $(Fabc)_2$ fragments derived from GPC-8 (Fig. 6). On the other hand, although Ig(1a)15.3 precipitates the $(Fab')_2$ and $(Fabc)_2$ fragments, it reacts poorly, if at all with the GPC-8 Fc fragment (Fig. 6). None of the hybridoma antibodies reacts with Fab fragments.

From these results it would appear that the allotypic determinant recognized by Ig(1a)15.3 is located in or near the hinge region of GPC-8. After this paper was submitted, however, we found that Ig(1a)15.3 reacts with the Fc fragment from another Igh-1a protein, a hybridoma-derived protein called 29-B.1 (34). Both GPC-8 and 29-B.1 Fc fragments were made with the same digestion procedure and both have the same Mr when analyzed by SDS-polyacrylamide gel electrophoresis, yet the allotypic determinant on GPC-8 recognized by Ig(1a)15.3 is lost after papain digestion, whereas the 29-B.1 Fc retains reactivity. In view of these results, a general conclusion on the location of this determinant on secreted Igh-1a proteins cannot be made.

The determinant on GPC-8 is not at the carboxy-terminus of the molecule, because the $(Fab')_2$ and $(Fabc)_2$ fragments from GPC-8 react with Ig(1a)15.3. The intact GPC-8 hinge region either contains the determinant or somehow affects reactivity with the determinant, located elsewhere on the molecule. This could be a result of a genetic difference between the two proteins. GPC-8 was derived from a BALB/c subline maintained in Australia which has been separated from the BALB/cN subline since

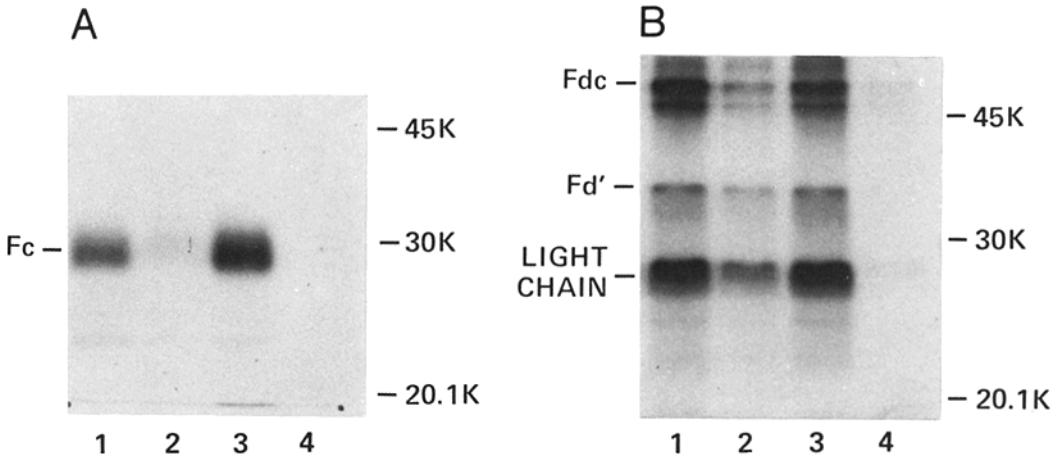

FIG. 6. Immunoprecipitation of Igh-1a immunoglobulin fragments with hybridoma antibodies. (A) shows the fragments precipitated from a mixture of ^{125}I-labeled Fab and Fc fragments (derived by limited proteolysis [with papain] of GPC-8, an Igh-1a myeloma protein) with Ig(1a)16.3 (lane 1), Ig(1a)15.3 (lane 2), Ig(1a)14.4 (lane 3), and Ig(4a)10.9 (lane 4). This is a 10% SDS-polyacrylamide slab gel run under nonreducing conditions. (B) shows the fragments precipitated from a mixture of $(Fab')_2$ and $(Fabc)_2$ fragments (derived by limited proteolysis [with *S. aureus* protease V8] of GPC-8 myeloma protein) with the same hybridoma antibodies described in (A). This is a 10% SDS-polyacrylamide slab gel run under reducing conditions. The heavy chain components of each fragment (see Fig. 5) and the light chain are indicated on the left side of the autoradiograph. Autoradiographic exposures were for 3 d with intensifying screens.

1952 (Dr. Noel L. Warner. Personal communication.). 29-B.1 was derived recently from the BALB/cN subline.

Removal of the N-Glcosidic-linked Oligosaccharide on Secreted IgG_{2a} Does Not Affect the Expression of the Ig(1a)15.3 Allotypic Determinant. We have already demonstrated that different *N*-glycosidic-linked oligosaccharide chains do not account for the difference in Mr and electrophoretic charge between membrane and secreted IgG_{2a}. Our results also demonstrate that the nonglycosylated secretory heavy chain precursor in 2PK3 cells treated with tunicamycin is precipitated with Ig(1a)15.3 (gel not shown). Thus the removal of the oligosaccharide chain on secreted IgG_{2a} does not affect the expression of the Ig(1a)15.3 allotypic determinant.

Nonglycosylated Membrane IgG_{2a} Heavy Chains Do Not Appear to Express Allotypic Determinants. We are not certain whether the nonglycosylated membrane molecule expresses the Ig(1a)15.3 determinant. It is conceivable that the membrane *N*-glycosidic-linked oligosaccharide masks this allotypic determinant. We have not been able to test this possibility because it appears that nonglycosylated membrane molecules are not precipitated with any of the hybridoma antibodies.

In untreated 2PK3 cell extracts, only a portion of the immunoglobulin precipitable with SaC is precipitable with hybridoma antibodies. Cell extracts that have been exhaustively cleared of immunoglobulin reactive with hybridoma antibodies still have immunoglobulin that is precipitable with SaC. This is true for membrane and secretory molecules that are precipitable with Ig(1a)14.4, which reacts with both molecules, and for secretory molecules precipitable with Ig(1a)15.3 (data not shown). A possible explanation for this may be that the expression of some allotypic and/or isotypic determinants are dependent on intracellular IgG tetramer assembly; protein A binding may not have such a prerequisite.

In tunicamycin-treated cell extracts, the reactivity of nonglycosylated secretory heavy chains with hybridoma antibodies is indistinguishable from their glycosylated homologues; however, the amount of nonglycosylated membrane heavy chain that is precipitable with Ig(1a)14.4, which precipitates some of the internal glycosylated membrane heavy chain, is small, if present at all, compared with SaC precipitation. (Fig. 2B shows the SaC precipitate of tunicamycin-treated cell extracts.) It appears that the expression of some allotypic determinants on membrane IgG_{2a} heavy chains and not secretory heavy chains is dependent on asparagine-linked glycosylation. We do not know the molecular basis of this result. It may be that glycosylation is important for the proper folding of the membrane heavy chain or the assembly of membrane IgG tetramer, and these processes are required for determinant expression.

One might expect that two distinct assembly pathways for membrane and secretory molecules are needed in a single cell that produces both molecules. These pathways would be dependent on structural differences (perhaps in the hinge region) between the membrane and secreted molecules. This would explain why mixed IgG_{2a} tetramers that consist of two light chains associated with one membrane and one secretory heavy chain have not been seen (Fig. 2A and B). Precedents for protein structure affecting assembly patterns have been described (37, 38).

Discussion

We have demonstrated that membrane and secreted IgG_{2a} from a monoclonal cell line are different on the basis of Mr, net charge, and allotypic determinants. The charge and molecular-weight differences are not a result of different *N*-glycosidic-linked oligosaccharides, but the possibility that the membrane molecule has additional serine- and/or threonine-linked glycosylation sites has not been excluded rigorously. Such *O*-glycosidic-linked oligosaccharides could account for the differences between the membrane and secreted heavy chains described here; however, the presence of additional sites for oligosaccharide attachment would suggest that membrane and secreted heavy chain molecules have primary-amino-acid-sequence differences.

The allotypic determinant recognized by Ig(1a)15.3 was shown not to be affected by removal of the *N*-glycosidic-linked oligosaccharide chain on secreted IgG_{2a} molecules. Because secreted IgG molecules are known to have a single asparagine-linked oligosaccharide chain, we are certain that the Ig(1a)15.3 allotypic determinant is not this carbohydrate structure. It still is conceivable however, that the asparagine-linked oligosaccharide on membrane heavy chains is sufficiently different that it masks this allotypic determinant. There are previous indications that membrane-associated immunoglobulins appear to lack allotypic and isotypic antigenic determinants found on secreted molecules (39–41). Some of these studies show that determinants near the carboxy terminus of the immunoglobulin apparently are inaccessible to antibody probes. The absence of the Ig(1a)15.3 determinant on detergent-solubilized molecules makes this an unlikely explanation for our results.

A recent study (42) indicated that IgG isolated from mouse spleen cell extracts behaves as though it contains a hydrophobic region. Our own preliminary data from charge-shift electrophoresis (43) experiments confirm this result. This would indicate that the membrane IgG_{2a} heavy chain we have described is an integral membrane protein.

Membrane IgM and IgD clearly contain hydrophobic structures that could anchor

them in membranes (43, 44);[2] however, there are no published primary amino acid sequence data demonstrating this extra hydrophobic segment. Although peptide-mapping studies demonstrate that there are differences between membrane and secreted IgM heavy chains (10), the location of these peptide differences has yet to be determined.

Our data, demonstrating a missing allotypic determinant on membrane IgG, suggest that a primary structural difference occurs in the Fc part of the molecule. Taken together with the evidence that membrane IgG may have a hydrophobic sequence anchoring it in the membrane, it would appear that there may be significant structural differences between membrane and secreted IgG_{2a} heavy chains, one within the Fc part of the molecule and the other near the carboxy-terminus (for which there is no direct evidence). This structural model would account for the molecular weight, charge, and allotypic differences between the two molecules. It also provides a structural basis for the faithful assembly of membrane and secreted tetrameric IgG molecules in the same cell.

Two other structures also could explain the differences described here. One is that the addition of a carboxy-terminal hydrophobic segment to membrane IgG_{2a} induces a conformational change in another region of the heavy chain, accounting for the absence of the Ig(1a)15.3 allotypic determinant. The other is that a primary structural difference in the membrane molecule induces a conformational change in the carboxy-terminus, exposing a hydrophobic region which then anchors the molecule in the membrane. It is conceivable that different membrane heavy chain homologues could adopt any of these structural strategies.

Structurally distinct membrane and secretory molecules of the same immunoglobulin heavy chain class could be generated in two ways: (*a*) there could be two distinct cistrons coding for membrane and secreted molecules; or (*b*) an alternate RNA splicing mechanism could yield similar but different messenger RNA (mRNA) from the same primary RNA transcript, which are translated into membrane and secreted heavy chains. From peptide mapping studies (10), it appears that the primary amino acid sequences of membrane and secreted IgM heavy chains are very similar (though not identical). Allotypic and isotypic determinants also are generally conserved between membrane and secreted molecules. If there are distinct cistrons for each molecule, we would expect more divergence in allotypy and isotypy between membrane and secreted products.

We feel that it is more likely that an alternate mRNA splicing mechanism generates both gene products from the same DNA sequence. Recently, the entire genomic sequence of the (secreted) IgG_{2b} (Igh-3a) heavy chain cistron was completed (45, 46). There is a 300 base intron separating the C_H1 and hinge exons that is large enough to accomodate an additional hinge region exon. An alternate hinge exon could be either entirely in the intron or overlap the secreted hinge exon. An overlapping sequence could be read in or out of frame, resulting in shared or different Fc sequences.

Simian virus 40 (SV40) genomic expression provides an excellent precedent for such an RNA splicing mechanism (47). Two early mRNA, arising in SV40-transformation code for two serologically and biochemically similar transformation (T) antigens. These have been shown to be derived from overlapping viral genomic DNA

[2] Goding, J. W., and L. A. Herzenberg. Biosynthesis of lymphocyte surface IgD in the mouse. *J. Immunol.* In press.

sequences (48, 49). Alternate RNA splicing may be a general mechanism for synthesizing two (or more) different, but nearly identical, proteins. Recently, a third SV40 early mRNA has been described that also shares T antigen-coding sequences (50). This mRNA represents an additional RNA splicing product that may code for the tumor-specific transplantation antigen (TSTA) found associated with membranes of SV40-transformed cells (50, 51). TSTA is serologically indistinguishable from T antigen (51). Examination of the SV40 genomic sequence that codes for this mRNA transcript has suggested that translation read out-of-phase at the 3′ end of the viral cistron (relative to the T-antigen-coding sequence) would yield a polypeptide with a hydrophobic carboxy-terminus (50). A similar mechanism could operate on immunoglobulin cistrons, yielding a membrane anchor for IgG heavy chains.

The difference between membrane and secreted IgG_{2a} also could play a key functional role in T cell regulation of antibody formation. B cell differentiation and antibody production is known to be regulated by both carrier-specific and Ig-specific helper T cells (52–57). The molecular basis for the interaction between Ig-specific T cells and Ig-bearing B cells has been a puzzle. It has always been difficult to imagine such a recognition process occuring in the presence of secreted molecules sharing the same antigenic determinants. Some of the recognition sites must also be genetically polymorphic, because allotype-specific recognition by T cells has been observed in allotype-heterozygous mice (56, 57). Polymorphic determinants unique to membrane immunoglobulin then might be the recognition sites by which such interactions occur.

Summary

We have demonstrated that there are structurally distinct membrane and secreted IgG_{2a} immunoglobulin molecules. The membrane heavy chain is both larger and more acidic than the secreted molecule. This difference is not a result of different *N*-glycosidic-linked oligosaccharide chains. The membrane heavy chain also is antigenically different from its secreted homologue. This is based on the fact that secreted IgG_{2a} molecules express an allotypic determinant absent on membrane molecules.

We discussed the genetic control and gene organization of membrane and secreted immunoglobulin heavy chain sequences and suggest mechanisms controlling the expression of the simian virus 40 genome as models for alternate gene expression of membrane and secreted heavy chain polypeptide chains from the same DNA sequence. The possible biological significance of the membrane immunoglobulin acting as a recognition site for regulatory T cells also is discussed. The difference between membrane and secreted immunoglobulin is proposed as a possible explanation for the manner in which T cells interact with IgG on memory B cells in the presence of a large excess of IgG present in body fluids.

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