The EMBO Journal vol.9 no.7 pp.2117 – 2124, 1990

Differences in glycoprotein complexes associated with IgM and IgD on normal murine B cells potentially enable transduction of different signals

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Communicated by I.Pecht

Studies presented here demonstrate that IgM and IgD molecules on normal murine B lymphocytes exist in different, noncovalently associated molecular complexes containing distinct but potentially related glycoproteins. The glycoproteins in these complexes, particularly those associated with IgD, show striking differences in various lymphoid organs and in X-linked immunodeficient (Xid) mice. These differences are due in part to post-translational processing. They apparently reflect the differential expression of the Ig-associated glycoproteins in the various B cell subpopulations and lineages and the differential distribution of the subpopulations and lineages in the various lymphoid organs. In addition, they reflect structural differences in the IgM and IgD complexes which, we suggest, permit differential signal transduction by IgM and IgD on the same B cell.

Key words: IgM/IgD/Ig-associated glycoproteins/lymphoid organs/Xid

Introduction

IgM and IgD have both been shown to function as antigen receptors on normal B lymphocytes (Sell and Gell, 1965; Pernis *et al.*, 1970; Maino *et al.*, 1975). However, like T cell receptors, the intracytoplasmic portions of these surface Ig (sIg) molecules appear to be too short to interact directly with intracellular proteins for signal transduction and endocytosis (Gough and Cory, 1986; Cambier and Ransom, 1987; Clevers *et al.*, 1988; Weiss, 1989). Thus, several laboratories have suggested that sIg molecules are associated with other proteins in a molecular complex that signals the binding of antigen (Hombach *et al.*, 1988; Sakaguchi *et al.*, 1988; Kishimoto and Hirano, 1989). Studies using immunoprecipitation and molecular cloning suggest that this complex may include the B34 and MB-1 proteins (Hombach *et al.*, 1988; Sakaguchi *et al.*, 1988). However, these proteins have not been detected on normal B cells.

The characterization of the Ig-associated proteins on normal B cells is complicated by the variable expression of sIg molecules on different kinds of B cells (Hardy *et al.*, 1982, 1984; Hayakawa *et al.*, 1984, 1985). FACS analyses have shown that virtually all mature B cells in the periphery express both IgM and IgD; however, the quantitative expression of these antigen receptors differs widely. B cells in certain subpopulations/lineages characteristically express 50 times as much IgM as IgD while B cells in other subpopulations/lineages consistently express IgD at much higher levels than IgM. Furthermore, the frequencies of the B cell subpopulations/lineages differ in spleen, lymph node and peritoneum and are strongly influenced by genetic elements such as the X-linked immunodeficiency (Xid) (Scher, 1982; Hardy *et al.*, 1983). Thus, to develop a comprehensive view of the proteins associated with IgM and IgD, we have examined the Ig complexes on B cells from different lymphoid organs in normal and Xid mice.

In studies presented here, we demonstrate a remarkable heterogeneity in the glycoproteins associated with IgM and IgD. In addition, we show that differences in the glycoproteins associated with each of these immunoglobulins are accompanied by known differences in IgM and IgD expression in the B cell subpopulations and lineages. This heterogeneity, which we show is due partly to post-translational processing and partly to primary structural differences, provides a basis for functional differences between IgM and IgD molecules on individual B cells and between the Ig molecules on different kinds of B cells.

Results

IgD is associated with at least three proteins

Since digitonin and CHAPS are mild detergents which lyze cells with less disruption of noncovalently associated membrane protein complexes than NP-40 (Oettgen *et al.*, 1986; Lanier *et al.*, 1987), we have used immunoprecipitation analysis after lysing cells with these detergents to identify IgD-associated proteins. Anti-δ precipitates from spleen cell lysates show the expected δ heavy chain, κ and λ light chain bands on SDS–PAGE (Figure 1). In addition, immunoprecipitates from digitonin and CHAPS lysates (but not from NP-40 lysates) show three new protein bands with relative molecular masses of 42 kd, 37 kd and 32 kd. These three new proteins, called D1, D2 and D3 respectively, are also detected when spleen cells are treated with cross-linking reagents dithiobis(succinimidylpropionate) (DSP) or dimethy-3, 3′-dithiobispropionimidate (DTBP) before NP-40 lysis. Isotype control antibody does not precipitate any species (data not shown).

NP-40 elution of the anti-δ precipitates prior to gel analysis demonstrates that the three proteins that co-precipitate with IgD are only weakly associated with IgD molecules. Usually, proteins immunoprecipitated from digitonin lysates are eluted with SDS sample buffer and then analyzed by gel electrophoresis (as above). However, eluting with NP-40 buffer before eluting with SDS sample buffer separates the precipitated proteins into two fractions (Figure 2A). Proteins that are weakly associated with the immunoprecipitated protein are readily released by NP-40 buffer and hence are eluted into the supernatant. In contrast, the immunoprecipitated protein itself remains in the residue and is subsequently eluted by SDS sample buffer. Thus, the three proteins that co-precipitate with IgD are extracted into the

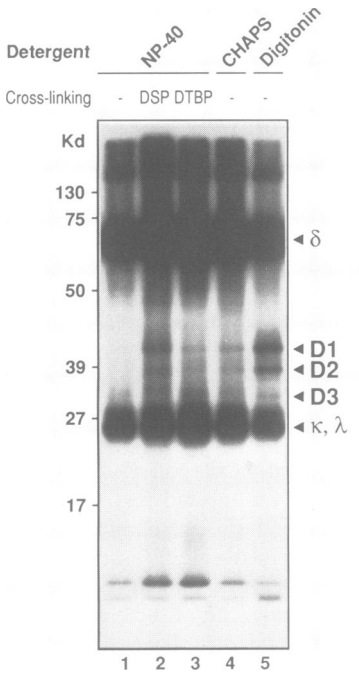

Fig. 1. SDS−PAGE analysis of anti-δ precipitates after cross-linking and lysis with different detergents. CBA/Ca spleen cells were lysed of red blood cells, surface labeled with ^{125}I by glucose oxidase and lactoperoxidase, and aliquoted. Two aliquots were treated with cross-linking reagents, DSP and DTBP, and lysed with NP-40 lysis buffer, the other three aliquots were each lysed with NP-40, CHAPS and digitonin lysis buffer. Anti-δ precipitates were analyzed by 12% SDS−PAGE under reducing conditions. **Lane 1**, NP-40 lysis; **lane 2**, cross-linked with DSP and then NP-40 lysis; **lane 3**, cross-linked with DTBP and then NP-40 lysis; **lane 4**, CHAPS lysis; and **lane 5**, digitonin lysis. Relative molecular masses were determined with prestained protein standards. The co-precipitated proteins are at 42 kd, 37 kd and 32 kd and are called D1, D2 and D3, respectively.

NP-40 buffer (Figure 2B, lane 2), while IgD is not eluted off by NP-40 buffer and remains in the residue until subsequent extraction by SDS sample buffer (Figure 2B, lane 3). No co-precipitated proteins are released by the NP-40 buffer from anti-δ precipitates of NP-40 lysates (Figure 2B, lane 5).

The IgD-associated proteins are further resolved into at least six spots, all of which have quite similar isoelectric points, in NEPHGE-2D gels (non-equilibrium pH gradient electrophoresis followed by SDS−PAGE) (Figure 3). The D1 (42 kd) and D3 (32 kd) species on SDS−PAGE each show up as a broad single spot, while the D2 (37 kd) species on SDS−PAGE gives rise to four to five discrete spots, distinguishable by their slightly different isoelectric points.

Diagonal gel analysis (nonreducing−reducing two dimensional SDS−PAGE) demonstrate that the D1 (42 kd) and the D3 (32 kd) species on SDS−PAGE are disulfide-linked homodimers in the IgD complex (Figure 4A). The D1 (42 kd) species migrates at ~84 kd in the nonreducing (first) dimension and does not align with any other spot in the reducing (second) dimension. The D3 (32 kd) species, which is not visible in Figure 4A but is visible with much longer exposures, similarly migrates at twice its size in the nonreducing dimension and does not align with any other spot in the reducing dimension. The D2 (37 kd) species also appears to be disulfide-linked homodimers in the native form. These proteins, which migrate at ~74 kd in the nonreducing

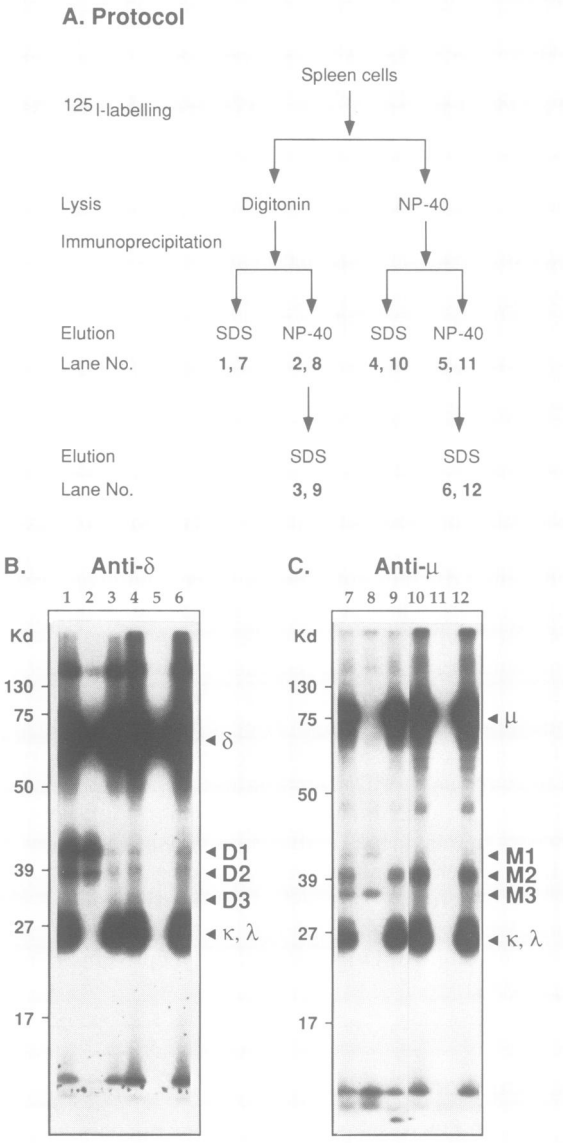

Fig. 2. SDS−PAGE analysis of anti-δ and anti-μ precipitates after lysis with digitonin or NP-40 and sequential elution with NP-40 and SDS. **A**, protocol. 2×10^7 spleen cells were ^{125}I-labeled and divided equally into two fractions, one fraction was lysed with digitonin lysis buffer and another with NP-40 lysis buffer. Then, digitonin and NP-40 lysates were each divided into two equal aliquots, one aliquot was precipitated with anti-δ monoclonal antibody and another aliquot was precipitated with anti-μ monoclonal antibody. Both anti-δ and anti-μ precipitates were again divided equally into two fractions. One fraction was eluted with SDS sample buffer to release all bound proteins (**lanes 1, 4, 7** and **10**). Another fraction was first eluted with NP-40 buffer to release weakly bound proteins (**lanes 2, 5, 8** and **11**), and was then eluted with SDS sample buffer to release tightly bound proteins (**lanes 3, 6, 9** and **12**). One third of each protein eluate was analyzed by 12% SDS−PAGE under reducing conditions. **B**, anti-δ precipitates, and **C**, anti-μ precipitates.

dimension, appear as partially overlapping spots of slightly different sizes in the reducing dimension.

Some IgD-associated proteins differ on B cells from different lymphoid organs

The differential distribution of B cell subpopulations and lineages in spleen, lymph node and peritoneal cavity (PerC) is accompanied by striking differences in the IgD-associated

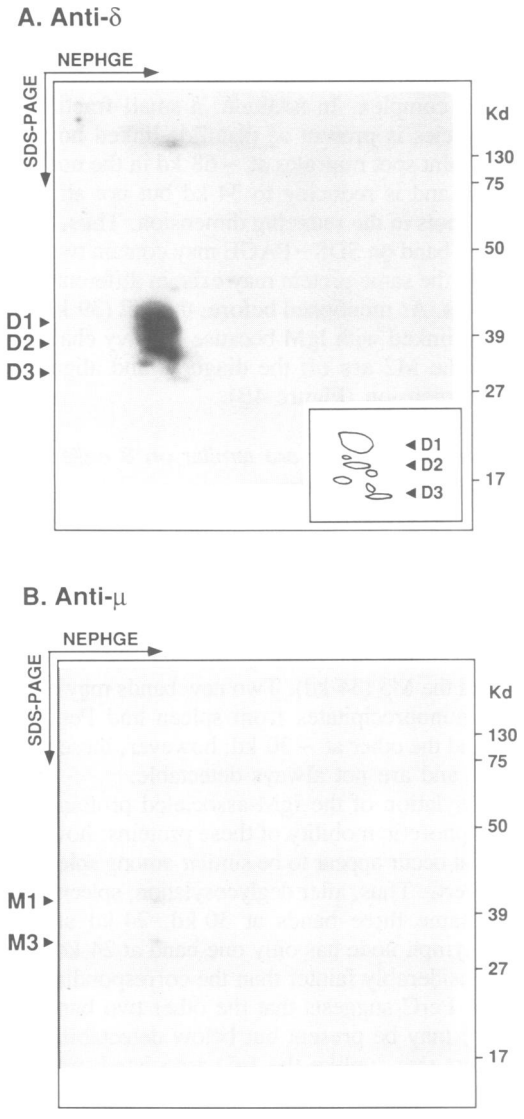

Fig. 3. NEPHGE-2D gel analysis of anti-δ and anti-μ precipitates. Anti-δ and anti-μ precipitates of digitonin lysates of CBA/Ca spleen cells were eluted with NP-40 buffer as in Figure 2. The eluted proteins were analyzed by non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and SDS−PAGE in the second dimension. **A**, anti-δ precipitates. Individual spots are schematically diagrammed in the insert. **B**, anti-μ precipitates.

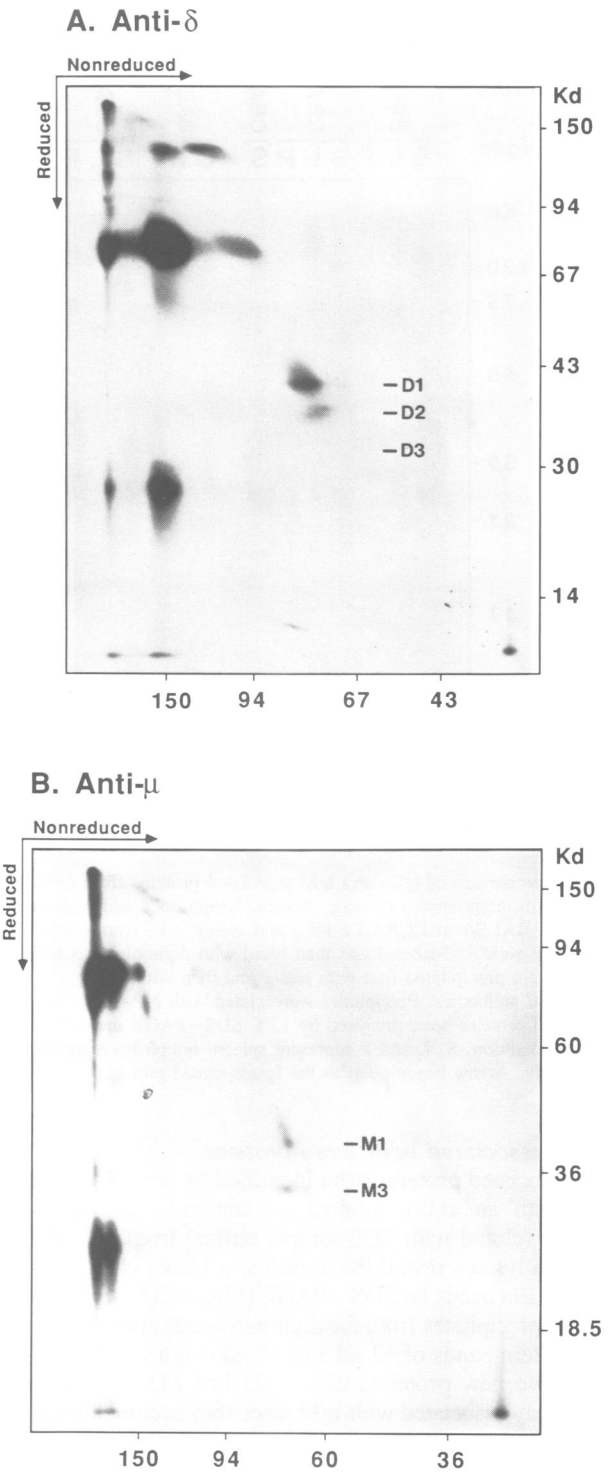

Fig. 4. Diagonal gel analysis of anti-δ and anti-μ precipitates. Anti-δ and anti-μ precipitates of digitonin lysates of CBA/Ca spleen cells were eluted with SDS sample buffer without reducing agent. The eluted proteins were analyzed by nonreducing SDS−PAGE in the first dimension and reducing SDS−polyacrylamide tube gel in the second dimension. **A**, anti-δ precipitates and **B**, anti-μ precipitates. The D3 (32 kd) spot from the anti-δ precipitate is visible only with much longer exposures.

proteins from these organs (Figure 5). Within the resolution of these gels, spleen, lymph node and PerC appear all to have the D1 (42 kd) and the D2 (37 kd) bands; however, the D3 (32 kd) band is undetectable from lymph node and PerC, even with three times longer exposures. Furthermore, lymph node cells appear to have a new band at 35 kd. This band is probably present at a low level in spleen but is obscured by the dominant D2 (37 kd) band.

Deglycosylation substantially reduces the size of the IgD-associated proteins from the various lymphoid organs; however, differences among these proteins are still detectable (Figure 6). Thus, deglycosylated proteins from spleen, lymph node and PerC show two bands at 30 kd and 24 kd. In addition, deglycosylated proteins from spleen give rise to a third band at 20 kd, which is undetectable from lymph node and is much less intense from PerC. Therefore, glycosylation may account for some of the differences in the IgD-associated proteins; primary structure differences also exist among these proteins.

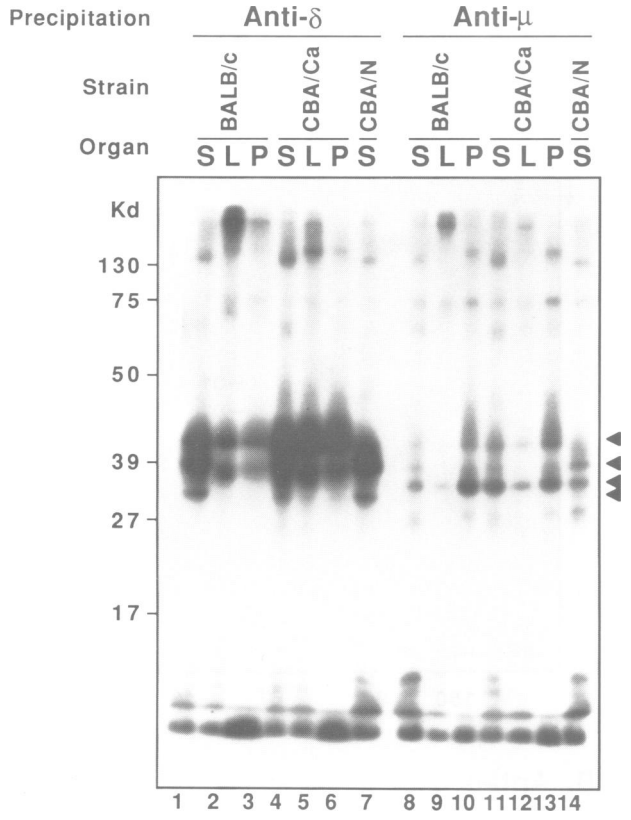

Fig. 5. Comparison of IgD- and IgM-associated proteins from different organs of different strains of mice. Spleen, lymph node and peritoneal cells from BALB/c and CBA/Ca mice and spleen cells from CBA/N (Xid) mice were ^{125}I-labeled and then lysed with digitonin lysis buffer. Lysates were precipitated first with anti-μ and then with anti-δ monoclonal antibodies. Precipitates were eluted with NP-40 buffer. The eluted proteins were analyzed by 12% SDS−PAGE under the reduced condition. S, L and P represent spleen, lymph node and PerC, respectively. Arrow heads point to the Ig-associated proteins.

IgM is associated with three proteins

IgM-associated proteins were identified by immunoprecipitation with an anti-μ monoclonal antibody. Immunoprecipitates (eluted with SDS sample buffer) from NP-40 and digitonin lysates reveal the expected μ heavy chain, κ and λ light chain bands on SDS−PAGE (Figure 2C). In addition, immunoprecipitates from the digitonin lysate give rise to two new protein bands of 42 kd and 34 kd (Figure 2C, lane 7). These two new proteins, called M1 and M3 respectively, are weakly associated with IgM since they are readily eluted by the NP-40 buffer (Figure 2C, lane 8). On the other hand, another species at 39 kd (M2), which is co-precipitated with IgM from both NP-40 and digitonin lysates, is not eluted off by NP-40 buffer; this is consistent with its disulfide linkage to IgM (Figure 2C and 4B). This protein may be equivalent to actin or the 30 kd species reported previously (Rosenspire *et al.*, 1986; Haustein and Von der Ahe, 1986). Alternatively, it could be a partially degraded product of the μ heavy chain of IgM.

NEPHGE-2D gel analyses of IgM-associated proteins reveal two spots with almost identical isoelectric points corresponding to the M1 (42 kd) and the M3 (34 kd) protein bands on SDS−PAGE (Figure 3B). Diagonal gel analyses reveal that the M1 (42 kd) and the M3 (34 kd) species migrate at ~75 kd in the nonreducing dimension and are aligned with each other in the reducing dimension (Figure 4B). They therefore exist as disulfide-linked heterodimers in the IgM complex. In addition, a small fraction of M3 (34 kd) species is present as disulfide-linked homodimers because a faint spot migrates at ~68 kd in the nonreducing dimension, and is reducing to 34 kd but not aligned with any other spots in the reducing dimension. Thus, the single M3 (34 kd) band on SDS−PAGE may contain two different proteins, or the same protein may exist in different disulfide-linked forms. As mentioned before, the M2 (39 kd) species is disulfide linked with IgM because μ heavy chain, κ light chain and the M2 are off the diagonal and aligned in the reducing dimension (Figure 4B).

IgM-associated proteins are similar on B cells from different lymphoid organs

IgM-associated proteins in B cell subpopulations and lineages in normal lymphoid organs appear to be less heterogeneous than the IgD-associated proteins (Figure 5). Within the resolution of SDS−PAGE gels, IgM-associated proteins from lymph node and PerC have the same bands as are found in spleen, i.e. the M1 (42 kd), the M2 (39 kd) (data not shown) and the M3 (34 kd). Two new bands may be present in the immunoprecipitates from spleen and PerC, one at ~36 kd and the other at ~30 kd; however, these bands are quite faint and are not always detectable.

Deglycosylation of the IgM-associated proteins changes the electrophoretic mobility of these proteins; however, the changes that occur appear to be similar among spleen, lymph node and PerC. Thus, after deglycosylation, spleen and PerC have the same three bands at 30 kd, 24 kd and 20 kd. Although lymph node has only one band at 24 kd, that this band is considerably fainter than the corresponding band in spleen and PerC suggests that the other two bands (30 kd and 20 kd) may be present but below detectability. These data suggest that, unlike the IgD-associated proteins, the IgM-associated proteins are the same in all lymphoid organs.

IgM-associated glycoproteins differ from IgD-associated glycoproteins in their native form

Since precipitations from the same lysate with anti-μ monoclonal antibody first and then with anti-δ monoclonal antibody, or *vice versa*, yield identical associated glycoproteins for each of the immunoglobulins, the two Ig-associated complexes are physically independent on the B cell surface. Furthermore, the proteins in these complexes are all different. M2, M3, D2 and D3 differ in size (Figures 2 and 5). M1 and D1 are the same size (42 kd); however, M1 is a heterodimer linked by disulfide bond(s) to M3 whereas D1 is a disulfide-linked homodimer (Figure 4). Thus, the complexes of IgM and IgD with their associated glycoproteins on B cells are self-contained and the glycoproteins in these complexes are distinguishable.

Deglycosylation reduces the size of all of the Ig-associated glycoproteins and removes most of the differences between glycoproteins associated with IgM and IgD (Figure 6). That is, IgM-associated and IgD-associated proteins from spleen and PerC give rise to three bands which are essentially equivalent (although the 20 kd band in spleen is less intense when obtained from IgM precipitates). Two of these bands are detectable in the IgD-associated proteins from lymph node and one is clearly detectable in the IgM-associated

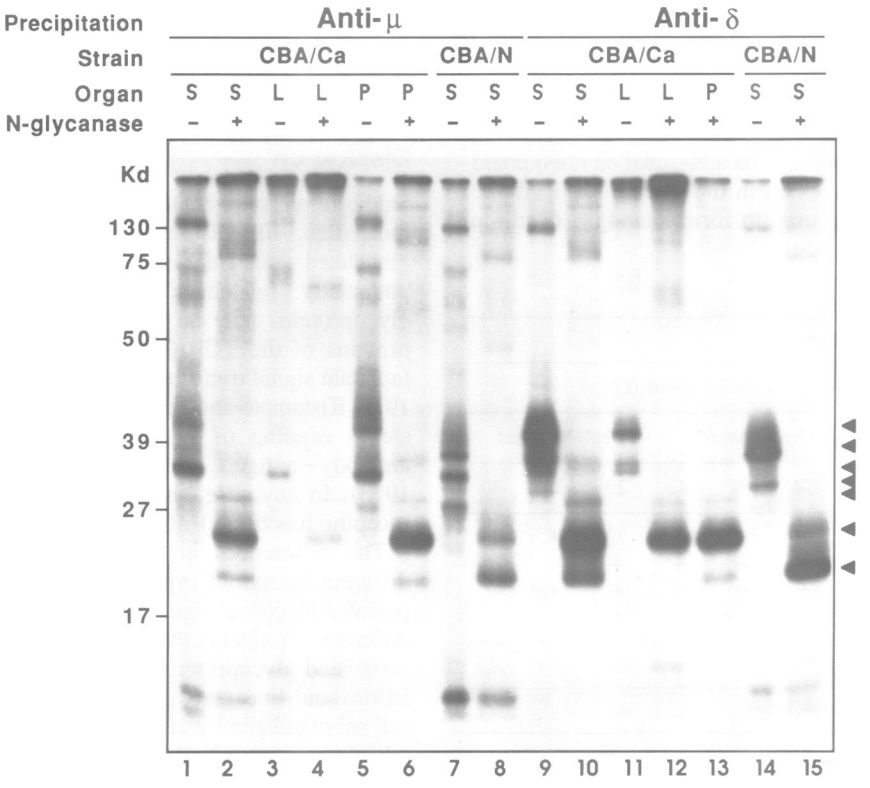

Fig. 6. SDS–PAGE analysis of deglycosylated IgM- and IgD-associated proteins. The same eluted proteins (not the same amount) from Figure 5 were treated with or without *N*-glycanase and analyzed by 12% SDS–PAGE gel under reducing conditions. S, L and P represent spleen, lymph node and PerC, respectively. **Lanes 2, 4, 6, 8, 10, 12, 13** and **15** are with *N*-glycanase, and **lanes 1, 3, 5, 7, 9, 11** and **14** are without the enzyme. Arrow heads point to the Ig-associated proteins both before and after deglycosylation.

proteins from lymph node (see above). The continued presence of three different-sized bands after deglycosylation demonstrates that at least three structurally distinct protein chains are utilized in the Ig-associated proteins. The similarity in size after deglycosylation, however, demonstrates that some of the differences in these glycoproteins are due to post-translational processing, such as glycosylation, disulfide bond formation and probably phosphorylation (Tedder and Schlossman, 1988).

Table I summarizes the characteristics of the IgM- and IgD-associated proteins from spleen, lymph node and peritoneum.

Some Ig-associated glycoproteins differ between normal and Xid mice

Within the resolution of the gel electrophoresis, the IgM- and IgD-associated glycoproteins from the same organ are identical in BALB/c and CBA/Ca mice. Furthermore, most of the IgM- and IgD-associated glycoproteins present in normal mice are found in spleen from CBA/N (Xid) mice, which are congenic with CBA/Ca but express the X-linked immunodeficiency (Xid). Surprisingly, however, spleens from CBA/N mice lack the 42 kd band that is associated with both IgM and IgD in all three lymphoid organs of normal mouse strains. Instead, a new band, particularly visible in the anti-μ precipitate, is present at ~36 kd. Only spleen cells were analyzed for CBA/N since lymph node and peritoneum in these immunodeficient animals do not yield enough cells for study.

Deglycosylation of the Ig-associated proteins from CBA/N spleen yields only two of the three bands that are generated from normal spleen (Figure 6). The third band (30 kd) is undetectable. In addition, the relative intensities of the two bands (24 kd) and 20 kd) that are present are reversed compared with those from normal spleen. Thus, the Xid defect, which is known to alter drastically B cell development, also has profound effects on the proteins associated with surface immunoglobulins. The effect may be partly on the post-translational processing of the Ig-associated proteins and/or distribution of B cell subpopulations and lineages.

The M3 (34 kd) protein may be identical to the previously characterized B34 protein

Various proteins have been shown to associate with IgM and/or IgD on murine spleen B cells and cell lines as summarized in Table II. Although the relationship of most glycoproteins we identified with previously characterized proteins is not evident, the M3 (34 kd) protein that we identified may be identical to the previously characterized B34 protein (Hombach *et al.*, 1988). Both proteins associate with IgM and are the same size in the glycosylated form (Figures 2 and 5). Futhermore, one of the three deglycosylated bands (24 kd) obtained from the IgM-associated glycoproteins is the same size as the deglycosylated B34 protein (Figure 6). However, B34 has been shown to be a disulfide-linked homodimer whereas only a small fraction of M3 is present as homodimers and most exists as disulfide-linked heterodimers with the M1 (42 kd) protein (Figure 4). Since this difference could be due to differences in the cell types on which these proteins are found (plasmacytomas versus normal mature B cells), there is still reason to believe that M3 and B34 are the same.

The M2 (39 kd) protein which is disulfide linked with IgM may be equivalent to actin or the 30 kd protein reported previously (Rosenspire *et al.*, 1986; Haustein and Von der Ahe, 1986). Thus, IgM may be a five or six chain covalently linked structure rather than the four chain covalently linked structure currently envisaged. The additional chain(s) could be linked to the μ heavy chain in the C_H3 domain via the cysteine residue which is used to form inter-IgM disulfide linkages in pentameric (secreted) IgM but has no defined linkage in the membrane form of the molecule (Putman *et al.*, 1973; Kehry *et al.*, 1979, 1982; Rogers *et al.*, 1980).

Table IA. IgM-associated proteins

		Associated Proteins (kd)					
		Glycosylated[a]			Deglycosylated		
Associated Ig	Organ	34	39[b]	42	20	24	30
IgM	lymph node	+	+	+	?	+	?
	peritoneum	+	+	+	+	+	+
	spleen	+	+	+	+	+	+
	Xid spleen	+	+	−	+	+	−

Table IB. IgD-associated proteins

		Associated Proteins (kd)					
		Glycosylated			Deglycosylated		
Associated Ig	Organ	32	37[c]	42	20	24	30
IgD	lymph node	−	+	+	−	+	+
	peritoneum	−	+	+	+	+	+
	spleen	+	+	+	+	+	+
	Xid spleen	+	+	−	+	+	−

M1, M2 and M3 are 42 kd, 39 kd and 34 kd IgM-associated proteins, respectively. D1, D2 and D3 are 42 kd, 37 kd and 32 kd IgD-associated proteins, respectively.
[a]There may be two additional proteins present in the spleen and PerC, one at ~36 kd and another at ~30 kd;
[b]the 39 kd (M2) protein is disulfide linked to IgM whereas the other proteins are noncovalently associated with IgM or IgD;
[c]the 37 kd (D2) species contains multiple species in NEPHGE-2D gel, each of which may have different intensities in different lymphoid organs.

Discussion

Studies presented here demonstrate that surface IgM and IgD molecules, like T cell receptors, exist in specifically associated molecular complexes containing distinct but potentially related glycoproteins. These Ig-associated glycoproteins may be functionally analogous to the CD3 proteins of the TCR/CD3 complex in that they serve to facilitate signal transduction by Ig molecules (Maino *et al.*, 1975; Kishimoto and Hirano, 1989). They may also participate in capping of Ig molecules and the endocytosis of antibody−antigen complexes (Unanue and Karnovsky, 1973). In any case, they are likely to be essential to the receptor function(s) of Ig molecules.

The differences in the Ig-associated glycoproteins in different lymphoid organs add another dimension to the potential functional significance of these glycoproteins. These differences reflect both the differential expression of the Ig-associated glycoproteins on the individual B cell subpopulations and lineages, and the differential distribution of B cell subpopulations and lineages in various lymphoid organs (Hardy *et al.*, 1982, 1984). Thus, for example, lymph node B cells belong mainly to a single subpopulation and their Ig-associated glycoproteins give a relatively simple band pattern. Spleen B cells, in contrast, are more heterogeneous and show a more complex pattern of the Ig-associated glycoproteins. The differences in Ig-associated glycoproteins could be responsible for some functional differences among the B cell subpopulations and lineages (Zan-Bar *et al.*, 1978; Hayakawa *et al.*, 1984, 1985).

The differential expression of the Ig-associated glycoproteins in B cell subpopulations and lineages may also account for the absence of the 42 kd glycoprotein from spleen B cells from CBA/N (Xid) mice. That is, these immunodeficient mice lack the predominant B cell subpopulation (IgM^{low}, IgD^{high}) found in spleen and lymph nodes from normal mice (Scher, 1982; Hardy *et al.*, 1983). Alternatively, the absence of the 42 kd glycoprotein may be due to an inability to produce or to process this protein

Table II. Comparison of IgM- and IgD-associated glycoproteins with other proteins

Species	Cell type	Protein	Size (kd)	Association	Linkage	Reference
murine	B	M1,M3	34,42(20,24,30)[a]	IgM	noncovalent	
	B	M2	39	IgM	disulfide	
	B	D1,D2,D3	32,37,42(20,24,30)	IgD	noncovalent	
	B	CD23	45(33)	IgM,IgD	noncovalent	Lee and Conrad (1985)
	B	46K	46	IgM	noncovalent	Koch and Haustein (1983)
	B	30K	30	IgM	disulfide	Haustein and Von der Ahe (1986)
	pre-B,B	MB-1	(24)	Ig(?)	?	Sakaguchi *et al.* (1988)
	pre-B	ω	18	μ	disulfide	Pillai and Baltimore (1987)
	pre-B	λ_5	−	μ(?)	?	Sakaguchi and Melchers (1986)
	pre-B	V_{preB}	−	μ(?)	?	Kudo and Melchers (1987)
	PC	B34	34(24)	IgM	noncovalent	Hombach *et al.* (1988)
	T	CD3	16−28(16,18)	TCR	noncovalent	Clevers *et al.* (1988)
chicken	B	actin	42	IgM	disulfide	Rosenspire *et al.* (1986)
human	pre-B	ΨL	16,18,22	μ	both	Kerr *et al.* (1989)

[a]size after deglycosylation; PC, plasmacytoma.

properly, perhaps for the same reason(s) that these mice fail to express a variety of other differentiation antigens found on normal B cells, e.g. Lyb-3, Lyb-5, Lyb-7 and Mls, or in plasmacytomas, e.g. the X-linked, lymphocyte-regulated (XLR) gene product (Ahmed *et al.*, 1977; Huber *et al.*, 1977; Subbarao *et al.*, 1979; Cohen *et al.*, 1985a, b). In any event, the absence of this protein band represents perhaps the most striking biochemical difference recognized to date between normal and Xid mice.

At the cellular level, the demonstration that IgM and IgD complexes on the same B cells are physically independent and apparently contain different glycoproteins offers new insights into the mechanism(s) of how mature B cells respond to antigen stimulation. Since IgM and IgD on an individual B cell have identical antigen combining sites and identical intracytoplasmic domains (Gough and Cory, 1986), there has always been a question as to whether these two receptors could transduce different signals (Sidman and Unanue, 1975; Zan-Bar *et al.*, 1978; Goodnow *et al.*, 1989). Our findings demonstrate clearly that two signals are possible and then what would determine which signal prevails when a B cell expressing both IgM and IgD antigen receptors encounters its cognate antigen.

Studies have shown that, by and large, individual B cells express significantly more IgM than IgD or *vice versa* (Hardy *et al.*, 1982, 1984; Hayakawa *et al.*, 1984). For example, the predominant B cell subpopulation in spleen expresses 50 times as much IgD as IgM. When these cells are presented with low doses of antigen, only a few IgD and essentially no IgM molecules will be occupied by antigen and the only signal transduced into the cells will be mediated by the IgD complex. In contrast, at high antigen doses, both the IgM and the IgD molecules will be occupied by antigen and either the IgM-mediated signal will prevail or the signals transduced by each of the antigen receptors will be arbitrated to define the B cell response. Thus, if IgM and IgD complexes can transduce different signals, the unequal expression of IgM and IgD antigen receptors on individual B cells constitutes a dual threshold mechanism that determines whether a B cell differentiates to antibody production or becomes non-responsive when stimulated by antigen.

Materials and methods

Mice

BALB/c, CBA/Ca and CBA/N (Xid) mice were bred in our laboratory animal facility and used at 3–5 months of age.

Monoclonal antibodies

The following monoclonal antibodies were used: AMS 9.1, a mouse IgG_{2a} anti-mouse δ heavy chain (Igh-5.1) (Stall and Loken, 1984); 331.1, a rat IgG_{2b} anti-mouse μ heavy chain (Kincade *et al.*, 1981); MAR 18.5, a mouse IgG_{2a} anti-rat κ light chain, used as second step antibody for 331.1 immunoprecipitation (Lanier *et al.*, 1982); UPC-10 (Sigma, St Louis, MO), a mouse IgG_{2a} anti-phosphocholine, used as an isotype control.

Surface labeling

Lymph node, peritoneal washout cells and red blood cell lysed spleen cells were washed three times with Dulbecco's PBS (D-PBS) free of Ca^{2+} and Mg^{2+} and once with D-PBS containing 10^{-6} M KI, and resuspended in D-PBS containing 10^{-6} M KI at $1-5 \times 10^7$ cells/ml. Cells were labeled with 0.2–1.0 mCi ^{125}I by the glucose oxidase/lactoperoxidase method (Keski-Oja *et al.*, 1977) at room temperature for 15 min, washed three times with D-PBS containing 0.1% NaN_3, and aliquoted. Each aliquot was lysed for 1 h at 0°C with 0.2–0.5 ml of one of the lysis buffers: NP-40 lysis buffer (1% NP-40, 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 10 mM iodoacetamide, 0.02% NaN_3, 1 mM EDTA and 1 mM PMSF); digitonin lysis buffer (1% digitonin (Calbiochem, San Diego, CA), 10 mM triethanolamine, pH 7.8, 150 mM NaCl, 10 mM iodoacetamide, 0.02% NaN_3, 1 mM EDTA and 1 mM PMSF) or CHAPS lysis buffer (5 mM CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfate}) (Calbiochem, San Diego, CA), 50 mM Tris–Cl, pH 8.0, 150 mM NaCl, 10 mM iodoacetamide, 0.02% NaN_3, 1 mM EDTA and 1 mM PMSF). For chemical cross-linking experiments, the cell aliquot was resuspended in D-PBS and treated with dithiobis(succinimidylpropionate) (DSP) or dimethy-3, 3′-dithiobispropionimidate (DTBP) (Pierce, Rockford, IL) at a final concentration of 1 mM for 1 h at 0°C. Treated cells were washed three times with D-PBS containing 0.1% NaN_3, and lysed in NP-40 lysis buffer for 1 h at 0°C. Cell lysates were centrifuged at 13 000 *g* for 5 min and used for immunoprecipitation.

Immunoprecipitation

Cell lysates were precleared twice with 25 μl of Sansorbin (Calbiochem) and aliquoted. Each aliquot was incubated with anti-δ, anti-μ or isotype control monoclonal antibodies, that had already absorbed to Pansorbin (Calbiochem), for 1 h at 0°C. Alternatively, the precleared lysates were first incubated with anti-μ monoclonal antibody and then with anti-δ monoclonal antibody for 1 h each at 0°C. Excess amounts of antibodies were used to saturate all protein A sites on Pansorbin and MAR 18.5 binding sites. Precipitates from digitonin and CHAPS lysates were washed four times with respective lysis buffers, and precipitates from NP-40 lysate were washed four times in NP-40 buffer (NP-40 lysis buffer with 0.375 M NaCl instead of 0.15 M). Bound proteins were eluted by incubation with 30 μl of SDS sample buffer (62.5 mM Tris–Cl, pH 6.8, 12.5% glycerol, 1.25% SDS and 0.001% bromophenol blue) without reducing agent for 5 min at room temperature and centrifuged at 10 000 *g* for 5 min. Alternatively, weakly bound proteins were eluted first by incubation with NP-40 buffer for 1 h at 0°C. After centrifugation at 10 000 *g* for 5 min, the pellets were incubated with SDS sample buffer to elute the tightly bound proteins.

Gel electrophoresis

Immunoprecipitates were boiled for 5 min in the presence of 100 mM 2-mercaptoethanol and analyzed by 12% SDS–PAGE using a discontinuous buffer system (Jones, 1980). Prestained protein standards (Bio-Rad, Richmond, CA) were used for relative molecular mass determinations. The precipitated proteins were also analyzed in two types of two dimensional gels. The first type is NEPHGE-2D gels with non-equilibrium pH gradient electrophoresis (NEPHGE) for the first dimension and 12% SDS–PAGE for the second dimension. The second type is diagonal gels with 10% polyacrylamide under nonreducing conditions for the first dimension and 12% SDS–PAGE under reducing conditions for the second dimension. Gels were dried and autoradiographed with Kodak XR films with intensifying screens at −70°C.

Deglycosylation

NP-40 eluted, Ig-associated proteins were boiled for 5 min in the presence of 0.5% SDS and 100 mM 2-mercaptoethanol. The solutions were adjusted to final concentrations of 0.2 M sodium phosphate, pH 8.6, 1.25% NP-40, and 10 U/ml *N*-glycanase™ (Genzyme, Boston, MA). The mixtures were incubated overnight at 37°C.

Acknowledgements

The authors thank Dr Aaron B.Kantor, Dr William G.Kerr and particularly Dr Lewis L.Lanier for helpful discussions and critical review of the manuscript. This work was supported by grants from NIH (CA 42509 and HD 01287).

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Received on February 19, 1990