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Two new proteins preferentially associated with membrane immunoglobulin D

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The IgM and IgD classes of antigen receptor can perform different functions on B cells. However, so far no class-specific components communicating with the cytoplasm have been found in the two antigen receptors. We have employed a new biotinylation protocol to search for intracellular membrane Ig-associated proteins. Here we describe two proteins of 29 and 31 kDa that are associated with membrane IgD and to some extent with membrane IgM. The membrane IgM molecule is associated specifically with three proteins of 32, 37 and 41 kDa. The purification and sequencing of the two mIgD-associated proteins revealed that they are novel proteins which are related to each other. These proteins may be the missing link between the antigen receptor and the cytoskeleton and may contribute to functional differences between membrane IgM and membrane IgD.

Key words: biotinylation/IgD/IgM/membrane-associated proteins

Introduction

Most mature B cells co-express two classes of membrane-bound immunoglobulin (mIg), mIgM and mIgD, with identical antigen-binding specificity. Immature B cells express only mIgM (Goding *et al.*, 1977; Vitetta and Uhr, 1977). Whether the two mIg classes have different functions on B cells is a matter of controversy. Several studies suggest that the engagement of mIgM and mIgD induces similar B cell responses (Sieckmann, 1980; Mond *et al.*, 1981; Brink *et al.*, 1992). This holds true for the early biochemical responses involving the activation of protein tyrosine kinases (PTK) (Gold *et al.*, 1990; Burkhardt *et al.*, 1991) and the hydrolysis of inositol phospholipids (Cambier *et al.*, 1987; Klaus *et al.*, 1987; Harnett *et al.*, 1989). Other studies, however, reveal functional differences between these two mIg classes and show that only mIgM but not mIgD can induce negative responses in B cells such as anergy and apoptosis (Cambier *et al.*, 1976; Vitetta *et al.*, 1976; Alés-Martínez *et al.*, 1988; Tisch *et al.*, 1988; Mongini *et al.*, 1989; Kim *et al.*, 1991, 1992; Carsetti *et al.*, 1993). The IgD knockout mice do not show a severe immunodeficiency, which suggests at least a partial redundancy in the function of mIgD and mIgM (Nitschke *et al.*, 1993; Roes and Rajewsky, 1993).

The B cell antigen receptor (BCR) is a complex between the mIg molecule and the Ig-α/Ig-β heterodimer. Ig-α and Ig-β are two related, glycosylated, transmembrane proteins encoded by the B cell-specific genes *mb-1* and *B29*, respectively (for reviews see Reth, 1992; DeFranco, 1993; Sakaguchi *et al.*, 1993). The Ig-α/Ig-β heterodimer plays an important role in the coupling of the BCR to several PTKs expressed in B cells (Clark *et al.*, 1992; Kim *et al.*, 1993a; Law *et al.*, 1993; Matsuo *et al.*, 1993; Sanchez *et al.*, 1993; Flaswinkel and Reth, 1994). Apart from a glycosylation difference between mIgM- and mIgD-associated Ig-α, the two mIg classes are coupled with the same Ig-α/Ig-β heterodimer (Campbell *et al.*, 1991; Venkitaraman *et al.*, 1991; Wienands and Reth, 1991a,b). Because mIgM and mIgD molecules also have an identical short cytoplasmic tail (Cheng *et al.*, 1982), both BCRs are expected to be alike in their cytoplasmic part. How IgM−BCR and IgD−BCR are able to transduce different signals inside the B cell has thus remained an enigma.

Apart from a few exceptions (Costa *et al.*, 1992; Cherayil *et al.*, 1993), mIgM is expressed on the cell surface only in association with the Ig-α/Ig-β heterodimer. In contrast, the mIgD molecule can be transported to the cell surface without the Ig-α/Ig-β heterodimer, as either a GPI-linked (Wienands and Reth, 1991a,b) or an α/β-independent transmembrane protein (Venkitaraman *et al.*, 1991; Kim, 1993; Williams *et al.*, 1993). The latter form of mIg was also found for the mIgG2b (Williams *et al.*, 1993) and mIgG2a molecules (Weiser *et al.*, 1994). Surprisingly, after cross-linking, the α/β-independent mIg is efficiently internalized, suggesting that it can interact with components of the cytoplasmic internalization machinery (Cherayil *et al.*, 1993; Kim, 1993; Weiser *et al.*, 1994). These findings could be accounted for by additional BCR components that link the receptor to the cytoskeleton. We have, therefore, searched for new mIg-associated molecules in mIg transfectants of the myeloma line J558L with a protein labelling protocol developed for this purpose. It allows the identification of components of protein complexes independently of their surface expression or rate of turnover. The mIg complexes were first bound to a sorbent and thereafter biotinylated on the sorbent (BOS). Using this protocol we have found two proteins that are associated with mIgD and to some extent with mIgM. Along with data reported in the accompanying paper by Terashima *et al.* (1994), our findings may explain the functional differences between the IgM−BCR and the IgD−BCR.

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Results

Detection of two proteins that are preferentially associated with mIgD

The myeloma lines J558Lδδm/mb-1 (Table I) and J558Lμm/mb-1 (Hombach *et al.*, 1990a) express on the cell surface large amounts of mIgD and mIgM, respectively. The two antigen receptors have the same antigen-binding site with specificity for the hapten 4-hydroxy-3-iodo-5-nitrophenyl (NIP). We employed NIP-coupled Sepharose beads to purify specifically mIg-associated proteins from Triton X-100 lysates of the J558L myeloma transfectants. After washing the beads with lysis buffer, all proteins bound to the beads were labelled with biotin (BOS protocol). Biotinylated proteins were then separated by non-reducing, 1-D SDS−PAGE and transferred to a nitrocellulose membrane. After incubating the nitrocellulose membrane with streptavidin-coupled horseradish peroxidase, the bound proteins were visualized by the enhanced chemiluminescence (ECL) detection system. The IgD−BCR complex precipitated from the Triton X-100 lysate of J558Lδδm/mb-1 cells contains, besides the mIgD molecule, two additional proteins of 31 and 29 kDa (Figure 1, lane 4).

Small amounts of the of 31 and 29 kDa proteins are also co-precipitated with the IgM−BCR in the lysate of J558Lμm/mb-1 cells (Figure 1, lane 2). The mIgM molecule, however, is mostly associated with two proteins of 32 and 37 kDa. These proteins were purified from J558Lμm/mb-1 and WEHI231 cells, sequenced and further characterized as described by Terashima *et al.* (1994). A third less abundant mIgM-specific protein of 41 kDa is also detected in precipitates of the IgM−BCR complex. These proteins do not bind non-specifically to NIP−Sepharose beads as the antigen NIP10−BSA inhibits to a large extent their co-precipitation (Figure 1, lanes 1 and 3).

When J558Lδδm/mb-1 and J558Lμm/mb-1 cells are surface-biotinylated (SB) prior to lysis, only the mIgM and mIgD molecules and none of the mIg-associated proteins are detected on the Western blot (Figure 1, lanes 5–8). This suggests that the mIg-associated proteins are exposed primarily to the intracellular rather than the extracellular space.

p29 and p31 are monomeric, non-glycosylated proteins

In non-reducing/reducing 2-D SDS−PAGE analysis, the two mIgD-associated proteins, p31 and p29, are located on the diagonal of the 2-D gel (Figure 2). They are, therefore, monomeric proteins and non-covalently associated with mIgD. We then asked whether p29 and p31 were glycosylated (Figure 3). After treatment of the

Table I. List of transfectants

Transfectants	mIg	α/β	Surface expression
J558Lμm/mb-1	V_H μ1 μ2 μ3 μ4 μm	+	+
J558Lδδm/mb-1	V_H δ1 δ3 δm	+	+
J558Lμ12μm	V_H μ1 μ2 μm	−	−
J558Lμ12δm	V_H μ1 μ2 δm	−	−
J558Lμ12Hm	V_H μ1 μ2 Hm	−	+

α/β association with the Ig-α/Ig-β heterodimer.

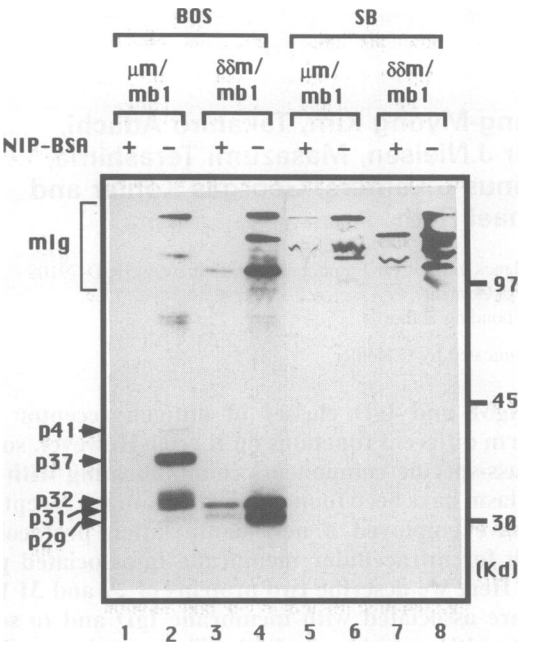

Fig. 1. Detection of novel mIgM- and mIgD-associated proteins by biotinylation on sorbent (BOS) but not by surface biotinylation (SB). Non-reducing, 1-D SDS−PAGE analysis. mIgM (lanes 1, 2, 5 and 6) and mIgD (lanes 3, 4, 7 and 8) were precipitated with NIP-coupled Sepharose beads from Triton X-100 lysates of J558Lμm/mb-1 and J558Lδδm/mb-1, respectively. The proteins were labelled by either BOS after immunoprecipitation (lanes 1–4) or surface biotinylation before cell lysis (lanes 5–8). In lanes 1, 3, 5 and 7 the specific binding of mIg to NIP−Sepharose was blocked by the addition of NIP-coupled BSA (NIP_{10}−BSA) (50 μg/ml) to the lysate before precipitation with NIP−Sepharose. Biotinylated proteins were detected on the Western blot using HRP−streptavidin and the ECL Western blotting detection system (Amersham).

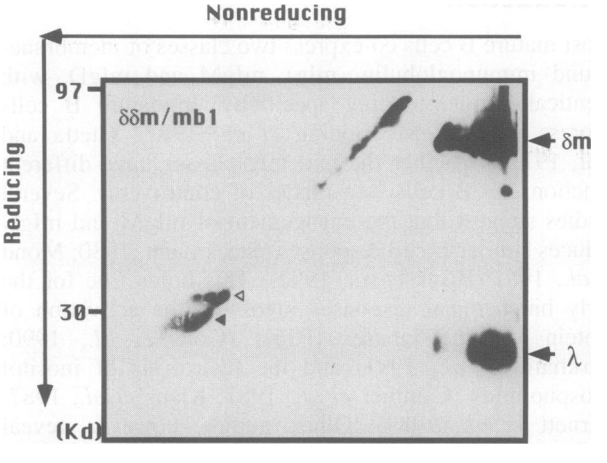

Fig. 2. 2-D SDS−PAGE analysis of mIgD-associated proteins. The mIgD molecules were precipitated with NIP-coupled Sepharose beads from Triton X-100 lysates of J558Lδδm/mb-1 cells. After labelling proteins by BOS, samples were subjected to 15% non-reducing/reducing, 2-D SDS−PAGE and Western blotting. The positions of the mIgD-associated proteins p29 and p31 are indicated by arrow heads. Biotinylated proteins were detected as mentioned in the legend to Figure 1.

purified IgD−BCR with *N*- and *O*-glycosidases, the molecular weight of mIgD decreases, whereas the molecular weights of p29 and p31 remain unchanged. Thus p29 and p31 are not glycoproteins. We refer here to these proteins as BCR-associated proteins (BAPs).

Together, these results indicate that the BAPs are different from the previously described Ig-α and Ig-β proteins. The latter proteins are heterodimeric transmembrane proteins which have a glycosylated extracellular Ig-like domain and are easily labelled by surface biotinylation (Kim *et al.*, 1993b).

Specific association between mIg and BAPs is determined by the transmembrane part of mIg

We next analysed the structural requirements of the class-specific binding of the mIg molecule to the BAPs. We constructed expression vectors encoding chimeric μ heavy chains for use in an exon-swapping experiment. Proteins μ12μm, μ12δm and μ12Hm have a common extracellular domain structure (V_H, C_H1 and C_H2) fused with the transmembrane (Tm) region of the mIgM, mIgD and H2-K^k molecules, respectively (Table I). The Tm region of each molecule comprises a short extracellular sequence (linker), the Tm part and a cytoplasmic tail of varying length. The vectors coding for these proteins were transfected into J558L myeloma cells. As has been reported for the μ12μm molecule (Hombach *et al.*, 1990b), the μ12δm chimeric protein is not associated with the Ig-α/Ig-β heterodimer (data not shown) and its transport to the cell surface is arrested in the endoplasmic reticulum (ER). The chimeric μ12Hm molecule has a very hydrophobic Tm part and is expressed on the cell surface without the Ig-α/Ig-β heterodimer.

After purification and labelling by the BOS protocol, the chimeric mIg molecules were examined for their association with BAPs. As shown in Figure 4, μ12μm is associated with BAP32, BAP37 and BAP41 (lane 2), while μ12δm is associated with BAP29 and BAP31 (lane 4). The chimeric μ12Hm molecule is not associated with any protein (Figure 4, lane 6). These data show that, in contrast to the Ig-α/Ig-β heterodimer, BAPs do not require the membrane-proximal C-domain for binding to the mIg molecule and are apparently associated only with the Tm region of mIg. As the mIgM and mIgD molecules have an identical cytoplasmic part (KVK), it seems that the linker and/or Tm part is essential for the class-specific association of mIg with BAPs. These data also show that the binding of the BAPs to the mIg molecule does not require the Ig-α/Ig-β heterodimer.

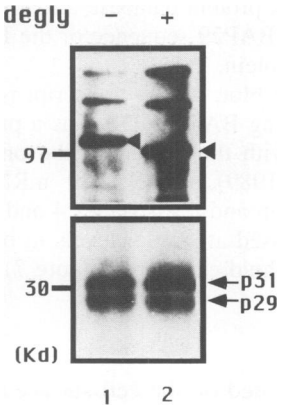

Fig. 3. BAP29 and BAP31 are non-glycosylated proteins. mIgD was immunoprecipitated with NIP-coupled Sepharose beads from Triton X-100 lysates of J558Lδδm/mb-1 cells. After labelling by BOS, sorbent-bound proteins were incubated with buffer alone (lane 1) or with buffer containing *N*- and *O*-glycosidases (lane 2). The position of the mIgD molecule is indicated by an arrow head. Biotinylated proteins were detected as described in the legend to Figure 1.

BAP29 and BAP31 are associated with mIgD on splenic B lymphocytes

BAP29 and BAP31 are associated with mIgD not only in δm-transfectants of the J558L myeloma line, but also in δm-transfectants of the B-lymphoma lines K46 and WEHI231 cells (data not shown). To test whether BAPs are also present in normal B lymphocytes, we examined mIgD molecules from splenic lymphocytes of NMRI mice. Proteins of similar size to that of BAP29 and BAP31 could be co-purified from a Triton X-100 lysate of 2×10^8 spleen cells with a monoclonal anti-IgD antibody (Figure 5A, lane 2) but not with a control antibody (lane 1).

Isolation and protein sequence analysis of BAP29 and BAP31

When mIgM or mIgD molecules are purified from lysates of $>1\times10^8$ J558Lμm/mb-1 or J558Lδδm/mb-1 cells, the associated BAPs can be detected with Coomassie brilliant blue R-250 on SDS−PAGE gels (Figure 5B). This protein staining method, which is not biased by the efficiency of labelling proteins, demonstrates that BAP37 and BAP32, as well as BAP31 and BAP29, are present in mIg precipitates roughly at a 1:1 ratio. It also shows that

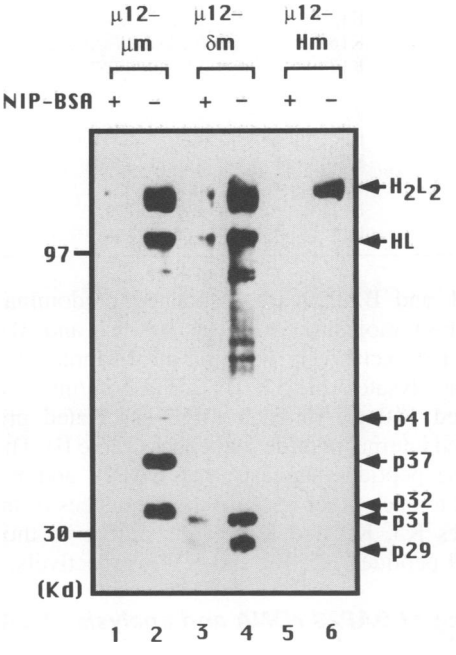

Fig. 4. The μm and δm transmembrane region is sufficient for the class-specific association with BAPs. The chimeric mIgs μ12μm (lanes 1 and 2), μ12δm (lanes 3 and 4) and μ12Hm (lanes 5 and 6) were precipitated with NIP-coupled Sepharose beads from the Triton X-100 lysates of J558Lμ12μm, J558Lμ12δm and J558Lμ12Hm, respectively. Purified proteins were labelled by BOS, subjected to 13.5% non-reducing 1-D SDS−PAGE, blotted and detected as described in the legend to Figure 1

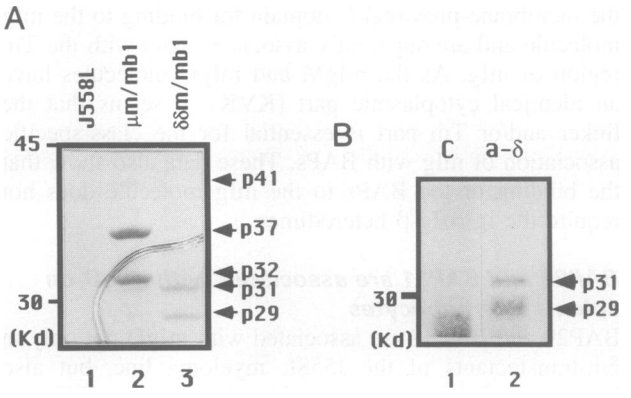

Fig. 5. (**A**) Copurification of BAP29 and BAP31 with mIgD from splenic lymphocytes. 2×10^8 spleen cells of NMRI mice were isolated by Ficoll–Paque centrifugation and lysed with Triton X-100. After preclearing of the lysate with protein G-coupled Sepharose beads, mIgD was precipitated from the cell lysate using the monoclonal anti-IgD antibody 10.4.22 and protein G-coupled Sepharose beads (lane 2). In a negative control (lane 1), instead of anti-IgD a monoclonal mouse anti-GST (glutathione-S-transferase) antibody was used. (**B**) Detection of the BAPs by Coomassie brilliant blue R-250 staining. Lysates of 2×10^8 cells of J558L (lane 1), J558Lμm/mb-1 (lane 2) and J558Lδδm/mb-1 (lane 3) were incubated with NIP–Sepharose and purified proteins were size separated by non-reducing 15% SDS–PAGE. The gel was stained with a 10.25% Coomassie brilliant blue R-250 solution and dried

Table II. Peptide amino acid sequence

BAP	Peptide	Sequence
31	K5	QSEGL TK
	K7	ENEAL AMQK
	K9	YMEEN ?T(L)
	K13	LQASV RGPSV K
	K16	EYDRL LEEHA K
	K17	LDIGN TEMKL EEN
29	K2	LIESK
	K3	KFMEE N(N)K
	K5	IQSER LSK
	K7	QN?VM
	K11	EYDRL LK
	K13	LGLRN DNAEE HLLEA ENK

BAP31 and BAP29 are associated predominantly with the mIgD molecule, whereas BAP37 and BAP32 are associated exclusively with the mIgM molecule.

Using lysates of 5×10^9 J558Lδδm/mb-1 cells, we prepared ~10 μg of each mIgD-associated protein and obtained internal peptide sequences (Table II). The analysis of these peptides suggests that BAP29 and BAP31 are related to each other because the sequences of the BAP31 peptides K5, K9 and K16 were similar to those of the BAP29 peptides K5, K3 and K11, respectively.

Cloning of BAP29 cDNA and analysis of BAP29 mRNA expression

Using degenerate primers, we amplified by PCR a 120 bp cDNA fragment of BAP29. This fragment was then used to isolate three independent cDNA clones from a cDNA library of the NIH/Swiss pre-B cell line 300-19P8 (Leclercq *et al.*, 1989). The sequence of one of the clones, a 1.7 kb cDNA, revealed an open reading frame of 720 bp that encodes a protein of 240 amino acids containing all six determined BAP29 peptide sequences (underlined in Figure 6). The coding region begins with a consensus ATG start codon. The predicted size of the protein encoded by this open reading frame is 28 kDa and thus close to the size of BAP29 as determined by SDS–PAGE. A search of the PIR protein database did not reveal a strong similarity of the BAP29 sequence or the BAP31 peptides to any known protein.

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   1 AAAGAAGCGCAGTGTAAGCCGGCGGCGGAGTGTGAAGAAAAAACAAAACAAAACGATGAC
                                                           M  T

  61 AATCCAATGGGCGGCGGTTGCCAGCTTTCTTTATGCTGAAATAGGACTCATTTTACTCTT
       I  Q  W  A  A  V  A  S  F  L  Y  A  E  I  G  L  I  L  L  F

 121 CTGTCTGCCCTTTATTCCTCCACAGAGATGGCAGAAGATTTTTTCATTTAGTGTCTGGGG
       C  L  P  F  I  P  P  Q  R  W  Q  K  I  F  S  F  S  V  W  G

 181 TAAGATTGCAAGTTTTTGGAACAAAGCTTTTCTTACCATTATAATACTATTGATCATTCT
       K  I  A  S  F  W  N  K  A  F  L  T  I  I  I  L  L  I  I  L

 241 GTTTCTAGATGCCGTGAGAGAAGTAAGAAAGTATTCGTCCACGAATGTTGTAGAGAAGAA
       F  L  D  A  V  R  E  V  R  K  Y  S  S  T  N  V  V  E  K  N

 301 CTCAGCCATCAGACCGAGTGCCTTTGAGCACACACAGATGAAGCTCTTCAGGTCTCAAAG
       S  A  I  R  P  S  A  F  E  H  T  Q  M  K  L  F  R  S  Q  R

 361 AAATCTGTATATTTCTGGATTCTCATTATTTTTTTGGCTAGTGTTGAGACGTCTGGTTAC
       N  L  Y  I  S  G  F  S  L  F  F  W  L  V  L  R  R  L  V  T

 421 CCTTATTACTCAGCTGGCAAAAGAAATAGCAAACAAAGGAGTGCTTAAAATTCAAGCAGA
       L  I  T  Q  L  A  K  E  I  A  N  K  G  V  L  K  I  Q  A  E

 481 AAATACCAATAAGGCTGCCAAGAAATTCATGGAAGAGAATGAAAAACTAAAATTGGGGCT
       N  T  N  K  A  A  K  K  F  M  E  E  N  E  K  L  K  L  G  L

 541 AAGAAATGACAATGCTGAAGAACACCTTTTGGAAGCTGAAAATAAAAAACTAATAGAAAG
       R  N  D  N  A  E  E  H  L  L  E  A  E  N  K  K  L  I  E  S

 601 CAAGGAGAACCTGAAAACCGAGCTGAAGAAGGCTTCCGATGCCCTTTTGAAGGCCCAAAA
       K  E  N  L  K  T  E  L  K  K  A  S  D  A  L  L  K  A  Q  N

 661 TGATGTCATGACTATGAAGATTCAGTCAGAGAGACTTTCTAAAGAGTATGACCGACTCCT
       D  V  M  T  M  K  I  Q  S  E  R  L  S  K  E  Y  D  R  L  L

 721 GAAAGAACACTCAGAACTTCAGAACCGTTTAGAGAAAGAGAAGAAGAAAGGCCTGTGAAC
       K  E  H  S  E  L  Q  N  R  L  E  K  E  K  K  K  G  L  *

 781 TAACTTTATAAAAGAAGACTGTAATACACCTTGTCAAAGTTCAGTTGAAACAAAGAAAGC
 841 TGTGCTAGCGCCTGCAGACCCGCACAGGCTATACAGTAGTGACATTTCCAAAATATTTGA
 901 TAATGTTTCAGATTTCAGATATAAAGTATGGATTTCAGCTCTTTTGGAAAATATAAGCAT
 961 GTTAAATACCATATTTACATATTGCTAATAGCATTGATATATGGTGGCTCTTTACCCATA
1021 AAAGGAAATTTTCATTTGCTAGCATTTACTTCCCAAATTAGATCTGAAGTTGCATGAAAC
1081 CATTATTAGCTTTGAAAGCTTTGATGTGTTTTTAGTAATAATCAGATGATGGTAGTGGTA
1141 ATGAGTAACCACAATTAGCCACTGCGCTTATCGAGAAGAGTCAGAGAACGGCGAAAACTA
1201 CGGCCTTAGGGCTCTTCAAGCCTCATGATTGTACAACCTACTCGAAATATGACAGCACTG
1261 TTGATAGTGTGTGTTGCTACTGTGCTTCTGTGCCTATAAGACTATTGATAATTTGTTTAG
1321 TCATATATGGTCAGTAGTCTGTCTTTTCAAGGTCATCTGCTAATAGAATGTATGCCCATT
1381 TGCAAAACTGTCATACCACTGAACTGAGCTTATGTTGCACTGTGGATTTCTCCACTGACA
1441 AAGCTTNNNNNNNNNCCCTAAAAGCAGCGCTTTTCTATTTGTGAAAGTTACTGATTGTA
1501 CTGGTATTACCTCAGTATTCATGCACTGGTATCATACAGATCTCTCTACTATGTTTCTAT
1561 ATTCTCAAAGNNAAATACAGGTTTTCATGCAAATATTCATGTAGAAGTAGTGAGTGTAAT
1621 AATATGATTTAGGTTATTTCAGTGTTTAACTGTGAAAAGCCAGGAATTC
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Fig. 6. Sequence of BAP29 cDNA. The predicted amino acid sequence is shown below the DNA sequence. The BAP29 peptide sequences determined are underlined. The sequence of mouse BAP29 is deposited with the EMBL database under accession number X78684.

On a Northern blot, a 2 kb transcript is detected in all tissues tested using BAP29 cDNA as a probe (Figure 7). In comparison with the signal of ribosomal protein S12 (Ayane *et al.*, 1989), the BAP29 mRNA is strongly expressed in brain and testis (lanes 4 and 6). The BAP29 mRNA is expressed at similar levels in both B-lymphoid and non-B-lymphoid cell lines (Figure 7).

Discussion

Proteins not exposed on the cell surface and with only a low turnover rate cannot be detected by either surface iodination/biotinylation or biosynthetic labelling. To overcome this problem, we have developed a new protein labelling protocol which is suitable for the detection of components of protein complexes. According to this protocol, protein complexes are first bound to a sorbent

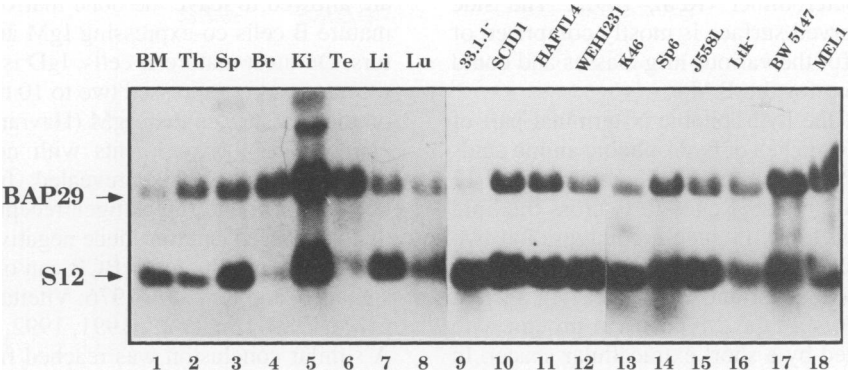

Fig. 7. BAP29 mRNA is expressed in many tissues and cell lines. Total RNA was isolated from the indicated sources, separated on denaturing 1% agarose gels, blotted to nitrocellulose and probed with either a BAP29 probe or, as a control, with a probe for mRNA of the ribosomal protein S12. Lanes 1–8 contain mRNA of bone marrow (BM), thymus (Th), spleen (Sp), brain (Br), kidney (Ki), testis (Te), liver (Li) and lung (Lu), respectively.

and subsequently biotinylated on the sorbent (BOS). Looking for new components of the mIgD antigen receptor we have identified the mIgD-associated proteins BAP29 and BAP31. These proteins are monomeric, non-glycosylated polypeptides which cannot be labelled on the cell surface. They therefore have features of intracellular proteins. The co-purification of BAP29 and BAP31, however, requires the transmembrane region of the mIgD molecule, suggesting that BAPs are also membrane proteins. The complex of BAP29/BAP31 with mIgD is found in lysates of B cell lines and normal splenic B cells.

Features of BAPs

The first 120 amino acids of BAP29 are mainly (63%) hydrophobic, whereas most of the C-terminal 120 amino acids are charged or polar (Figure 8). In agreement with the biochemical data, no N-linked glycosylation sites are found in the BAP29 sequence. A computer-based search did not reveal a strong similarity to any protein sequence in the PIR database. Four of the six BAP31 peptides, however, have a sequence similar to that of the BAP29 protein. This shows that BAP31 and BAP29 are closely related proteins. The mIgD molecule is thus bound by a pair of related proteins.

The situation of the mIgD molecule is thus quite similar to that of the mIgM molecule which is also bound by a pair of related proteins, prohibitin/BAP32 and the prohibitin-like protein BAP37 (Terashima *et al.*, 1994). In biochemical terms, the BAPs of mIgD and mIgM have similar features. They are all small (29–37 kDa), monomeric, non-glycosylated proteins, which cannot be labelled on the cell surface and which are associated specifically with the linker and/or Tm part of the mIg molecule. Yet in the primary sequence there is no obvious homology between the BAPs of mIgD and mIgM. This lack of sequence homology does not exclude the possibility that BAPs have a similar overall structure. Indeed, all sequenced BAPs have a hydrophobic N-terminus, and according to the Chou–Fasman calculations (data not shown) BAP29 and prohibitin/BAP32 have a similar distribution of α-helical regions. Furthermore, as judged from their biochemical behaviour, each pair of BAPs should have a membrane anchor and a part that is exposed to the cytoplasm.

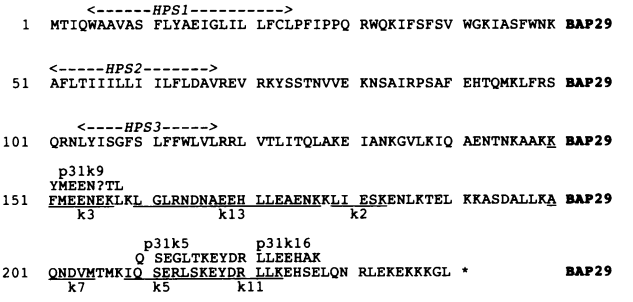

Fig. 8. Comparison of the amino acid sequence of BAP29 with the sequence of some of the BAP31 peptides. The sequences of BAP29 peptides are underlined and the peptide name is written below the sequence. The sequence of BAP31 peptides is given on top of the BAP29 sequence. The hydrophobic stretches (HPS1–S3) in the N-terminal part of BAP29 are indicated by arrows. The amino acid sequence is given as single-letter code.

Structural requirements for the association of BAP and mIg

What is the structural basis for the class-specific binding of the mIg and BAP molecules? Our exon-swapping experiment shows that the class-specific binding of the BAPs is solely determined by the sequence encoded by the Tm exons of mIg. This sequence consists of a negatively charged extra-cellular linker, the Tm and the cytoplasmic part. The mIgD and mIgM molecules have quite different linkers of 27 and 13 amino acids, respectively. In the Tm part, the two mIg molecules differ in 11 out of 25 residues. The short cytoplasmic tail (Lys-Val-Lys) is identical in both mIg molecules. Thus, assuming that mIgD and BAP29 proteins are in direct contact with each, BAP29 must penetrate the membrane to bind the mIgD molecule in a class-specific manner. The finding that the mIgD–BAP complex can be isolated from the membrane fraction of J558L even after a high salt wash (data not shown) supports the notion that BAP29 and BAP31 are integral membrane proteins.

The 25 amino acids of the Tm part of mIg are thought to cross the lipid bilayer as an α-helix. Fourteen of these amino acids are conserved in all mIg classes. As most of these amino acids lie on one side of the α-helix, this side was proposed as an interaction surface between the mIg

and the Ig-α/Ig-β heterodimer (Reth, 1992). The side opposite to this conserved surface is mostly comprised of amino acids specific for the various mIg classes and could form a binding surface for the BAPs.

An examination of the hydrophobic N-terminal part of BAP29 reveals three stretches of hydrophobic amino acids (Figure 8, HPS1−HPS3). The first two contain 20 and 17 amino acids and would be large enough to cross the lipid bilayer in an α-helical form. In such a configuration two models can be envisaged. First, BAP29 is a type 1 Tm protein with HPS1 as a leader and HPS2 as a Tm anchor. Alternatively, BAP29 is a type 2/type 1 Tm protein with two Tm parts separated by a short extracellular spacer. In both models, the bulk of the BAP29 protein, and in particular its charged C-terminus, is located in the cytoplasm. We favour the second model because HPS1 does not fulfil all the criteria of a leader sequence and the cleavage of the leader would make BAP29 much smaller than its estimated size. The Tm parts of multi-barrel membrane proteins are sometimes hard to identify because their sequence can contain several polar or even charged amino acids (Barclay *et al.*, 1993). Given these considerations, it is possible that BAP32 and BAP37 are also membrane proteins with two Tm parts in their N-terminal regions (Terashima *et al.*, 1994). At present it is not clear whether BAP32/BAP37 and BAP29/BAP31 form non-covalent dimers, or if these proteins only meet when they bind to the mIg molecule. Antibodies against the BAPs are required to answer these questions. Such antibodies would also be useful to demonstrate the co-localization of mIg and the BAPs in the plasma membrane or inside the cell, a result which would exclude the possibility that these proteins bind to each other and to mIg only in the cell lysate.

BAPs and the Ig-α/Ig-β heterodimer

The formerly discovered Ig-α and Ig-β proteins are quite different from the mIgD-associated BAPs. Ig-α and Ig-β are heterodimeric glycoproteins with an extracellular Ig-like domain that can be easily biotinylated on the cell surface (Kim *et al.*, 1993b). Both the Tm part and the membrane-proximal C-domain of the mIg molecule are required for the binding of the Ig-α/Ig-β heterodimer to mIg (Hombach *et al.*, 1990a). Apart from a glycosylation difference between IgD- and IgM-associated Ig-α (Wienands *et al.*, 1990; Campbell *et al.*, 1991; Venkitaraman *et al.*, 1991), all mIg classes are associated with the same Ig-α/Ig-β heterodimer (Venkitaraman *et al.*, 1991). Thus, in contrast to the binding of the BAPs, the binding of the Ig-α/Ig-β heterodimer to the mIg molecule is not class-specific.

The Ig-α and Ig-β proteins have a cytoplasmic tail of 61 and 48 amino acids, respectively, and both sequences carry a TAM/ARH1 motif. The predicted cytoplasmic region of BAP29 has no obvious signalling motifs. Another striking difference between these proteins is that Ig-α and Ig-β are only found in B cells, whereas BAPs are expressed in all tissues and cell lines tested. This suggests that BAPs have a more general role.

Functional differences between mIgD and mIgM

Immature B cells which express exclusively IgM antigen receptors are subjected to selection processes before they are allowed to leave the bone marrow. They then become mature B cells co-expressing IgM and IgD antigen receptors. On most mature B cells, IgD is the dominant antigen receptor and is expressed two to 10 times more abundantly on the cell surface than IgM (Havran *et al.*, 1984; Carsetti *et al.*, 1993). Experiments with normal and transgenic mouse B cells have revealed functional differences between IgM and IgD antigen receptors, and it is believed that while IgM can transduce negative and positive signals into the B cell, the IgD−BCR can only transduce positive signals (Cambier *et al.*, 1976; Vitetta *et al.*, 1976; Mongini *et al.*, 1989; Kim *et al.*, 1991, 1992; Carsetti *et al.*, 1993). A similar conclusion was reached from studies on the B-lymphoma lines WEHI231 and CH33, which co-express IgM−BCR and IgD−BCR (Alés-Martínez *et al.*, 1988; Tisch *et al.*, 1988). However, it has not been possible so far to correlate the functional differences of the two receptors with structural differences. Indeed, characterization of both receptors has revealed identical cytoplasmic parts of the mIg molecules and the Ig-α/Ig-β heterodimer.

If the class-specific BAPs are really associated with the mIg molecule in the plasma membrane, they may offer a solution to this puzzle. BAPs may modulate the signalling and/or internalization function of the Ig-α/Ig-β heterodimer. The mIgD molecules expressed on the cell surface without Ig-α/Ig-β heterodimer can still be efficiently internalized (Kim, 1993). As these α/β-less mIgD molecules are still associated with BAP31/BAP29 (data not shown), the BAPs may be part of the ill-defined cellular internalization machinery. Alternatively, BAPs may control a unique signalling pathway. The identity of the mIgM-associated BAP32 with prohibitin (Terashima *et al.*, 1994), which was purified based on its growth-arresting property (McClung *et al.*, 1989; Nuell *et al.*, 1991; Roskams *et al.*, 1993), is interesting in view of the fact that only the IgM−BCR but not the mIgD−BCR is thought to promote growth-arresting signals. The function of the mIgD-associated BAPs remains unclear so far. Knockout mice lacking the mIgD−BCR are not drastically immunodeficient. This result suggests that mIgM may functionally replace mIgD in these mice. The observation that the mIgM molecules can also bind small amounts of the IgD-associated BAPs may explain the phenotype of these mice. Further studies on the unknown function of the BAPs may be instrumental in understanding the specific roles of each mIg class during the different stages of B cell development. As BAPs are expressed in many different tissues, they may function as universal linker molecules between a heterogeneous set of receptors with components of the cytoskeleton.

Materials and methods

Construction of vectors

The mIgD expression vector pSVgδδm was constructed as a variation of our previously used pSVδm vector (Wienands *et al.*, 1990). A *Bgl*II−*Kpn*I fragment (Cδ), including the Cδ1, CδH and Cδ3 exons of mouse IgD, and a *Bam*HI−*Bam*HI fragment (δm), containing the transmembrane exons encoding the extracellular linker, transmembrane and cytoplasmic parts of mIgD, were obtained from a λ phage clone Charon 28-257.3 (Tucker *et al.*, 1980; Cheng *et al.*, 1982). An *Eco*RI−*Eco*RI fragment (VE), coding for the variable region of the heavy chain derived from a hybridoma B1-8 (Bothwell *et al.*, 1981) and the IgH enhancer, was obtained from pSVμm (Reth *et al.*, 1987) and its

internal *Bam*HI site was filled in. After subcloning and a change of restriction sites, the three fragments were ligated and the product (δδm) was inserted into the *Eco*RI site of the expression vector pSV2gpt to obtain pSVgδδm.

The expression vector pVCH12μm was made by deleting the Cμ3 and Cμ4 domains from pSV-Vμ1 (Neuberger, 1983). In pVCH12δm and in pVCH12Hm, the μm transmembrane exons of pVCH12μm are replaced by the δm transmembrane exons and by a fragment (Hm) coding for the linker, transmembrane and cytoplasmic parts of the H-$2K^k$ class I MHC protein (Arnold *et al.*, 1984), respectively.

Cell lines

J558L is a myeloma cell line producing λ1 light chains but no heavy chains (Oi *et al.*, 1983). J558Lμm/mb-1 has been described previously (Hombach *et al.*, 1990a). Cells were cultured in RPMI-1640 medium supplemented with 15% FCS, 2 mM L-glutamine, 2×10^{-5} M 2-mercaptoethanol and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Transfection of the expression vectors was performed by electroporation using 10^7 J558L cells and 10 μg of vector DNA (Potter *et al.*, 1984). Transfectants were selected in the presence of 1 μg/ml mycophenolic acid (Sigma).

Biotinylation on sorbent (BOS)

One million cells were lysed in 0.5 ml of Triton X-100 lysis buffer (pH 8.0) containing 20 mM Tris−HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM PMSF. After a 10 min incubation, insoluble material was removed by centrifugation at 10 000 *g* for 10 min at 4°C and mIg was precipitated with NIP-coupled Sepharose beads from the supernatant of the lysate. The immunoprecipitate was washed once with lysis buffer, twice with PBS and incubated in 100 μl PBS containing 0.5 mg/ml Sulfo-NHS-Biotin (Pierce) at room temperature for 15 min. The immunoprecipitate was washed twice with lysis buffer and subjected to SDS−PAGE and Western blotting.

Surface biotinylation (SB)

Surface biotinylation was performed as described previously (Kim *et al.*, 1993b) with some modifications. Cells (2×10^7) were washed twice with PBS and incubated in 1 ml of PBS containing 0.5 mg/ml of Sulfo-NHS-Biotin (Pierce) at room temperature for 15 min. Free succinimide groups were then blocked by the addition of 5 ml of non-supplemented medium at room temperature for 5 min. Then the cells were washed twice with PBS and lysed in 1 ml of ice-cold Triton X-100 lysis buffer. After incubation for 10 min on ice, the supernatant was cleared by centrifugation at 10 000 *g* for 10 min at 4°C and specific mIg was precipitated with NIP-coupled Sepharose beads.

Deglycosylation

The immunoprecipitate was resuspended in 10 μl of denaturing buffer composed of 20 mM Tris−malate buffer (pH 7.0) and 0.25% SDS, and boiled for 3 min. Then 40 μl of incubation buffer composed of 20 mM Tris−malate buffer (pH 7.0), 1% NP-40, 0.5 mM PMSF and 4 μl each of *N*-glycosidase F (0.2 mU/μl) and *O*-glycosidase (0.5 mU/μl) (Boehringer Mannheim) were added to the sample, and the mixture was incubated at 37°C overnight.

Protein sequence and cloning of the cDNA of BAPs

The mIgD receptor complex was affinity purified from 5×10^9 J558Lδδm/mb-1 cells. Gel slices containing size-separated protein bands of BAP29 and BAP31 were digested with lysC protease and the peptides purified and sequenced on Beckman machines by the Toplab protein sequence service (Munich, Germany). The peptide sequence was used to design a panel of degenerate primers which were used in the PCR to isolate a 120 bp BAP29 fragment. Full-length BAP29 cDNA clones were isolated from a 300-19P8 NIH-Swiss pre-B cell cDNA phage library subcloned in Bluescript and sequenced by ALF (Pharmacia).

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