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The specificity of association of the IgD molecule with the accessory proteins BAP31/BAP29 lies in the IgD transmembrane sequence

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Mature B cells co-express on their cell surface two classes of antigen receptor, the IgM and IgD immunoglobulins. The structural and functional differences between the two receptor classes are poorly understood. Recently two proteins of 29 and 31 kDa (BAP29 and BAP31) have been described that are preferentially associated with membrane IgD but only weakly with membrane IgM. We describe here the cloning of full-length murine and human *BAP31* cDNAs encoding proteins of 245 and 246 amino acids respectively. The two BAP31 proteins are 95% identical. The *BAP31* gene is ubiquitously expressed in murine tissues and is located on the X chromosome in both mouse and man. The murine BAP31 protein has 43% sequence identity to murine BAP29. Both proteins have a hydrophobic N-terminus and an α-helical C-terminus which ends with a KKXX motif implicated in vesicular transport. By a mutational analysis we have identified amino acids in the transmembrane sequence of the δm chain that are critical for binding to BAP31/BAP29. A structural model of the BAPs and their potential functions are discussed.

Keywords: antigen receptor/BAP/B cell/immunoglobulin/ transmembrane protein

Introduction

The B cell antigen receptor (BCR) is a multimeric structure composed of the membrane-bound immunoglobulin molecule (mIg) and the Ig-α/Ig-β heterodimer (reviewed in Reth, 1992; DeFranco, 1993; Cambier *et al.*, 1994). While the mIg molecule serves as the ligand binding component of the receptor, the non-covalently associated Ig-α/Ig-β heterodimer has been shown to be the signal transduction unit of the BCR. Ig-α (CD79a) and Ig-β (CD79b) are two glycosylated transmembrane proteins of the Ig superfamily and are encoded by the B cell-specific genes *mb-1* (Sakaguchi *et al.*, 1988) and *B29* (Hermanson *et al.*, 1988) respectively. Both proteins carry an immunoreceptor tyrosine-based activation motif (ITAM) which plays an important role in communication between the BCR and protein tyrosine kinases. Assembly of the mIg molecule and the Ig-α/Ig-β heterodimer into a multimer is in most cases required for surface expression of the BCR (Hombach *et al.*, 1990). The Ig-α/Ig-β heterodimer is also implicated in internalization of the BCR and its transport to intracellular compartments where antigen processing takes place (Patel and Neuberger, 1993).

The Ig-α/Ig-β heterodimer is associated with all classes (IgM, IgD, IgG, IgA and IgE) of the BCR (Venkitaraman *et al.*, 1991). Therefore signaling differences between the IgM BCR and IgD BCR (Kim and Reth, 1995) cannot reside in the Ig-α/Ig-β heterodimer. We have recently found four BCR-associated proteins (BAPs) in mouse B cells which bind to the mIg molecule in a class-specific manner. Two of these proteins (BAP32 and BAP37) are specifically associated with the IgM BCR (Terashima *et al.*, 1994), while the other two (BAP29 and BAP31) bind preferentially to the IgD BCR (Kim *et al.*, 1994). Experiments with chimeric receptors have shown that specific binding of BAPs to mIg molecules requires only the presence of the sequences encoded by the transmembrane exons of the μ or δ genes. The 25 amino acid transmembrane sequence of these two classes of heavy chains differ from each other at 11 positions.

All four BAPs are non-glycosylated membrane proteins. BAP32 is the murine homolog of a previously identified rat protein called prohibitin (for a review see McClung *et al.*, 1995). Injection of prohibitin mRNA into fibroblasts inhibits their progression through the cell cycle (McClung *et al.*, 1989). Somatic mutation of the human prohibitin gene has been found in some breast cancers, suggesting that it acts as a tumor suppressor gene (Sato *et al.*, 1992). In ovarian tumors, however, no mutations of the prohibitin gene have been found (Cliby *et al.*, 1993). BAP37 has 47% sequence identity with BAP32 (prohibitin) and both proteins are ubiquitously expressed. mIgD-associated BAP29 is only weakly related (15% sequence identity) to the mIgM-associated proteins (BAP32/BAP37). However, BAP29 shares structural features with the latter proteins, such as a hydrophobic N-terminus and a C-terminal half with a predicted α-helical structure. BAP29 is also ubiquitously expressed in murine tissues and cell lines. Partial peptide sequences from BAP31 suggested that this protein is related to BAP29. We report here the cloning of murine and human *BAP31* cDNA. At the protein level the murine sequences of BAP31 and BAP29 are 43% identical. The BAP31/BAP29 proteins bind preferrentially to the IgD BCR and we have identified critical amino acids in the transmembrane sequence of the δm chain which mediate this binding.

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       GGGATGAGTTTGCAGTGGACTACAGTTGCCACCTTCCTCTACGCAGAGGTCTTTGCTGTGTTGCTT  mBap31
       AGGATGACTCTGCAGTGGACTGCAGTTGCCACCTTCCTCTATGCGGAGGTCTTTGTTGTGTTGCTT  hBap31
          M  S  L  Q  W  T  T  V  A  T  F  L  Y  A  E  V  F  A  V  L  L
          M  T  L  Q  W  T  A  V  A  T  F  L  Y  A  E  V  F  V  V  L  L

 67    CTCTGCATTCCCTTCATTTCTCCAAAAAGATGGCAGAAGGTTTTAAATCCCGGCTGGTG  mBap31
 67    CTCTGCATTCCCTTCATTTCTCCTAAAAGATGGCAGAAGATTTTCAAGTCCCGGCTGGTG  hBap31
       L  C  I  P  F  I  S  P  K  R  W  Q  K  V  F  K  S  R  L  V
       L  C  I  P  F  I  S  P  K  R  W  Q  K  I  F  K  S  R  L  V

127    GAGTTGGTAGTGACCTATGGCAACACTTTCTTTGTGGTTCTCATCGTCATCCTTGTACTG  mBap31
127    GAGTTGTTAGTGTCCTATGGCAACACCTTCTTTGTGGTTCTCATTGTCATCCTTGTGCTG  hBap31
       E  L  V  V  T  Y  G  N  T  F  F  V  V  L  I  V  I  L  V  L
       E  L  L  V  S  Y  G  N  T  F  F  V  V  L  I  V  I  L  V  L

187    TTGGTTATTGATGCTGTACGAGAGATCCTGAAATACGATGATGTGACAGAAAAGGTGAAC  mBap31
187    TTGGTCATCGATGCCGTGCGCGAAATTCGGAAGTATGATGATGTGACGGAAAAGGTGAAC  hBap31
       L  V  I  D  A  V  R  E  I  L  K  Y  D  D  V  T  E  K  V  N
       L  V  I  D  A  V  R  E  I  R  K  Y  D  D  V  T  E  K  V  N

247    CTCCAGAACAATCCAGGTGCCATGGAGCACTTCCACATGAAGCTTTTCCGTGCTCAGAGG  mBap31
247    CTCCAGAACAATCCCGGGGCCATGGAGCACTTCCACATGAAGCTTTTCCGTGCCCAGAGG  hBap31
       L  Q  N  N  P  G  A  M  E  H  F  H  M  K  L  F  R  A  Q  R
       L  Q  N  N  P  G  A  M  E  H  F  H  M  K  L  F  R  A  Q  R

307    AATCTCTATATTGCTGGCCTTTCCTTGCTGCTGTCCTTCCTGCTTAGACGCCTGGTGACT  mBap31
307    AATCTCTACATTGCTGGCTTTTCCTTGCTGCTGTCCTTCCTGCTTAGACGCCTGGTGACT  hBap31
       N  L  Y  I  A  G  L  S  L  L  L  S  F  L  L  R  R  L  V  T
       N  L  Y  I  A  G  F  S  L  L  L  S  F  L  L  R  R  L  V  T

367    CTCATCTCCCAGCAGGCCACACTGCTGGCCTCCAATGAAGCCTTTAAAAAGCAGGCAGAA  mBap31
367    CTCATTTCGCAGCAGGCCACGCTGCTGGCCTCCAATGAAGCCTTTAAAAAGCAGGCGGAG  hBap31
       L  I  S  Q  Q  A  T  L  L  A  S  N  E  A  F  K  K  Q  A  E
       L  I  S  Q  Q  A  T  L  L  A  S  N  E  A  F  K  K  Q  A  E

427    AGTGCCAGTGAGGCGGCCAAGAAATACATGGAGGAGAATGATCAGCTAAAGAAGGGAGCT  mBap31
427    AGTGCTAGTGAGGCAGCCAAGAAGTACATGGAGGAGAATGACCAGCTCAAGAAGGGAGCT  hBap31
       S  A  S  E  A  A  K  K  Y  M  E  E  N  D  Q  L  K  K  G  A
       S  A  S  E  A  A  K  K  Y  M  E  E  N  D  Q  L  K  K  G  A
                                     K-9

487    GCCGAGGATGGAGACAAGTTGGATATTGGGAATACTGAAATGAAGTTAGAGGAG...AAC  mBap31
487    GCTGTTGACGGAGGCAAGTTGGATGTCGGGAATGCTGAGGTGAAGTTGGAGGAAGAGAAC  hBap31
       A  E  D  G  D  K  L  D  I  G  N  T  E  M  K  L  E  E  .  N
       A  V  D  G  G  K  L  D  V  G  N  A  E  V  K  L  E  E  E  N
                                       K-17

544    AAGAGCCTGAAGAATGACCTGAGGAAGCTAAAAGATGAGCTGGCCAGCACCAAGAAAAAA  mBap31
547    AGGAGCCTGAAGGCTGACCTGCAGAAGCTAAAGGACGAGCTGGCCAGCACTAAGCAAAAA  hBap31
       K  S  L  K  N  D  L  R  K  L  K  D  E  L  A  S  T  K  K  K
       R  S  L  K  A  D  L  Q  K  L  K  D  E  L  A  S  T  K  Q  K

604    CTTGAGAAAGCTGAAAACGAGGCTCTGGCTATGCAGAAGCAGTCTGAGGGCCTTACCAAA  mBap31
607    CTAGAGAAAGCTGAAAACGAGGTTCTGGCCATGCGGAAGCAGTCTGAGGGCCTCACCAAG  hBap31
       L  E  K  A  E  N  E  A  L  A  M  Q  K  Q  S  E  G  L  T  K
       L  E  K  A  E  N  E  V  L  A  M  R  K  Q  S  E  G  L  T  K
                      K-7                          K-5

664    GAATATGACCGCCTGCTAGAAGAACATGCCAAACTGCAGGCATCAGTACGTGGTCCCTCA  mBap31
667    GAGTACGACCGCTTGCTGGAGGAGCACGCAAAGCTGCAGGCTGCAGTAGATGGTCCCATG  hBap31
       E  Y  D  R  L  L  E  E  H  A  K  L  Q  A  S  V  R  G  P  S
       E  Y  D  R  L  L  E  E  H  A  K  L  Q  A  A  V  D  G  P  M
                K-16                          K-13

724    GTCAAGAAGGAGGAGTAAAGGCTTGGTGTTTCCCTGCCTGCCGCTGGCTTCTACCTGACC  mBap31
727    GACAAGAAGGAAGAGTAAGGGCCTCCTTCCTCCCCTGCCTGCAGCTGGCTTCCACCTGGC  hBap31
       V  K  K  E  E  *
       D  K  K  E  E  *
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Fig. 1. Nucleotide sequence of the mouse and human *BAP31* cDNA. The start and stop codons of the open reading frame are shown in bold. The deduced amino acid sequence is shown below the nucleotide sequence and the previously determined sequences of the murine BAP31 peptides (K5–K17) are underlined. The nucleotide sequence data of the murine and human *BAP31* cDNA has been deposited in the EMBL Database under accession Nos X81816 and X81817 respectively.

Results

Cloning of BAP31 cDNA and analysis of BAP31 mRNA expression

We have previously co-purified the BAP31 protein together with the mIgD molecule and obtained the sequence of five internal BAP31 peptides (Kim *et al.*, 1994). We designed a pair of degenerate primers derived from the sequences of two of these peptides (K-9 and K-16) and used them in a PCR to amplify DNA fragments from the cDNA libraries of J558L myeloma, mouse spleen and mouse bone marrow cells. These fragments were subcloned into a plasmid vector and sequenced. A 200 bp fragment contained the coding sequence for the BAP31 internal peptides K-17, K-7 and K-5, indicating that this fragment was amplified from *BAP31* cDNA. After removing a 30 bp repetitive sequence (628–658 in Figure 1) with 95% identity to the human minisatellite pMS43A sequence (Armour *et al.*, 1989) we used 150 bp of this fragment as a probe to screen a total of 8×10^5 phage clones of a J558L cDNA library.

Five positive *BAP31* phage clones were isolated, the largest of which carried a 1.2 kb insert which we subcloned and sequenced. The 5′ part of the sequence contains an ATG start codon which is the beginnning of an open reading frame of 735 bp (Figure 1). This reading frame encodes a protein of 245 amino acids containing all five previously determined internal BAP31 peptide sequences. By Edman degradation of the purified BAP31 protein we obtained the partial N-terminal sequence xSxQWTTxAT,

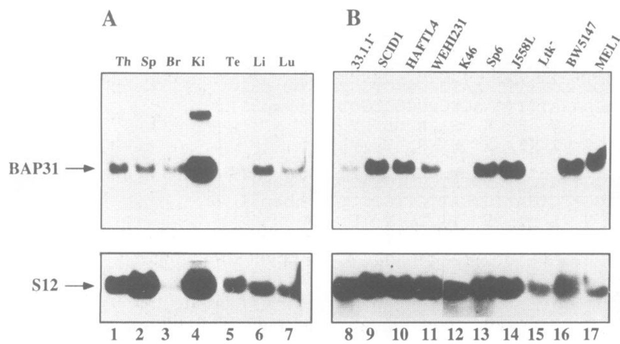


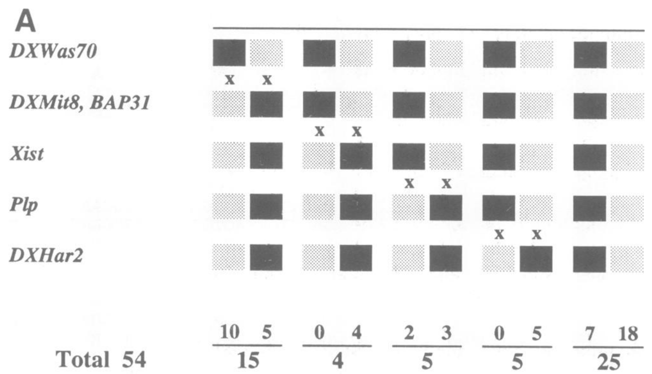
Fig. 2. Northern blotting analyses of *BAP31* gene transcripts from various tissues and cell lines. (**A**) Lanes 1–7 contain total RNA of thymus (Th), spleen (Sp), brain (Br), kidney (Ki), testis (Te), liver (Li) and lung (Lu) respectively. (**B**) Lanes 8–17 contain total RNA of Abelson murine leukemia virus-transformed pre-B cell lines 33.1.1$^-$, SCID1 and HAFTL4, the B cell lymphoma cell lines WEHI231 and K46, the hybridoma cell line Sp6, the myeloma cell line J558L, the fibroblast cell line Ltk$^-$, the thymoma cell line BW5147 and the erythrocyte leukemia cell line MEL1 respectively. Blots were hybridized with a *BAP31* cDNA probe (upper panels) or a probe for ribosomal protein S12 mRNA (lower panels).

which matches to the N-terminus of the cDNA-encoded protein. It is therefore likely that the cloned cDNA encodes the murine BAP31 protein. The predicted size of the protein encoded by the open reading frame is 27.935 kDa and is thus slightly smaller than the experimentally determined size of BAP31 (i.e. 31 kDa).

Expression of the *BAP31* gene was analyzed by Northern blotting using total RNA from various mouse tissues and cell lines and the complete *BAP31* cDNA as probe. A 1.5 kb *BAP31* transcript is detected in all tissues and cell lines tested (Figure 2). The transcript of the S12 ribosomal protein (Ayane *et al.*, 1989) was used as an internal standard in this analysis. Although the *BAP29* and *BAP31* genes are both expressed ubiquitously, their expression patterns differ from each other in that *BAP29* mRNA (Kim *et al.*, 1994) is more abundant in brain and testis than *BAP31* mRNA (Figure 2A, lanes 3 and 5). *BAP31* mRNA was also expressed in all cell lines tested (Figure 2B).

Chromosome localization of the mouse BAP31 gene

The chromosomal position of the mouse *BAP31* gene was determined by interspecific backcross analysis between two mouse strains, C57BL/6 and *Mus spretus*. To identify restriction fragment length polymorphisms (RFLPs) in the vicinity of the *BAP31* gene C57BL/6 and *M.spretus* DNAs were digested with several restriction enzymes and analyzed by Southern blot hybridization with a *BAP31* probe. *Pst*I digestion of these DNAs revealed a RFLP between the two strains in that the *BAP31* probe hybridized with a 6.0 kb *Pst*I fragment in C57BL/6 and a 5.2 kb *Pst*I fragment in *M.spretus* DNA. Therefore, we used *Pst*I in a haplotype analysis of interspecific backcross mouse DNA (Figure 3A). Known microsatellite polymorphism markers between BL/6 and *M.spretus* were then used to determine the map position of the *BAP31* gene. This analysis showed that, like its human counterpart, the mouse *BAP31* gene lies on the X chromosome. Sixty-nine backcross mice were analyzed without detecting



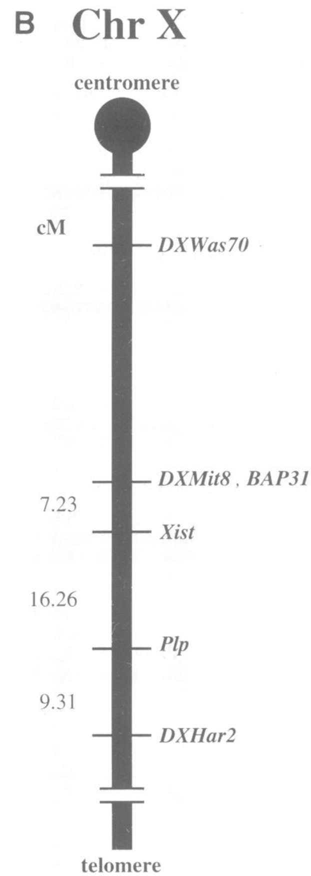


Fig. 3. Chromosomal localization of the mouse *BAP31* gene. (**A**) Haplotype analysis of C57BL/6×*M.spretus* interspecific backcross progeny carrying recombinations between the mouse microsatellite markers *DXWas70* and *DXHar2* in the vicinity of the *BAP31* gene locus. Each column represents the chromosome identified in the (C57BL/6×*M.spretus*)×C57BL/6 or *M.spretus* backcross progeny that was inherited from the F1 parent. The black squares show the presence of both *M.spretus* and C57BL/6 alleles and the shaded squares show the presence of either the C57BL/6 or *M.spretus* allele. The locus order was determined by minimizing the number of observed recombination breakpoints. (**B**) Linkage map of the proximal region of mouse chromosome X constructed on the basis of the interspecific backcrosses. The genetic distances between two neighboring loci are given in centimorgans at the left of the chromosome map.

recombination between the *BAP31* gene and the X chromosomal marker *DXMit8*, suggesting that these loci are close to each other (Figure 3B). The genetic order and distances around the *BAP31* locus are: Cen-*DXWas70* 27.8 ± 6.1 cM *DXMit8*, *BAP31* 7.4 ± 3.6 cM *Xist* 9.3 ± 3.9 cM *Plp* 9.3 ± 3.9 cM *DXHar2*a.

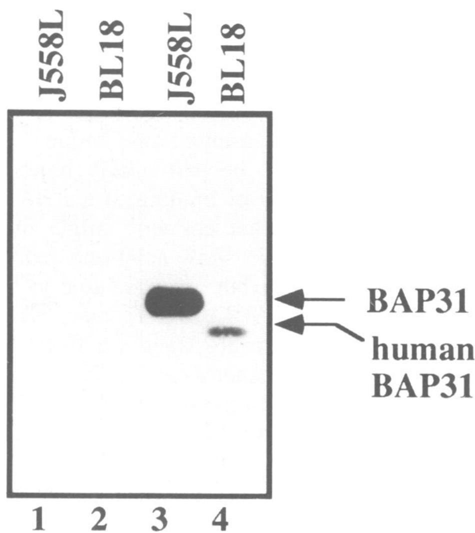


Fig. 4. Western blot analysis of mouse and human BAP31 protein. Total Triton X-100 cell lysates of murine myeloma J558L (lanes 1 and 3) and human B-lymphoma BL18 (lanes 2 and 4) cells were subjected to 15% non-reducing SDS–PAGE and Western blotting. The anti-mouse BAP31 antiserum used for detection of BAP31 protein was pre-cleared with Sepharose beads coupled either with GST–BAP31 fusion protein (lanes 1 and 2) or with GST alone (lanes 3 and 4). Bound anti-BAP31 antibodies were detected by the ECL system using a HRP-coupled goat anti-rabbit IgG antiserum.

Table I. Association of the different BAPs with wild-type and mutated mIg molecules

Heavy chain[a]	Percent association[b]	
	With BAP 29/31	With BAP 32/37
δAμBC	12 ± 4	7 ± 5
μAδBC	44 ± 1	0
μABδC	38 ± 9	0
δABC	102 ±2	0
δm	100	0
μm	3 ± 2	100
δ-D1	21 ± 1	0
δ-D2	81 ± 3	0
γ2am	0	0

[a]The transmembrane sequences of the wild-type and mutated δm heavy chains are shown in Figure 5.
[b]The numbers are mean values (see Materials and methods) derived from two independent transfectants, each analyzed in three different experiments. For the δABC heavy chain only one transfectant was analyzed.

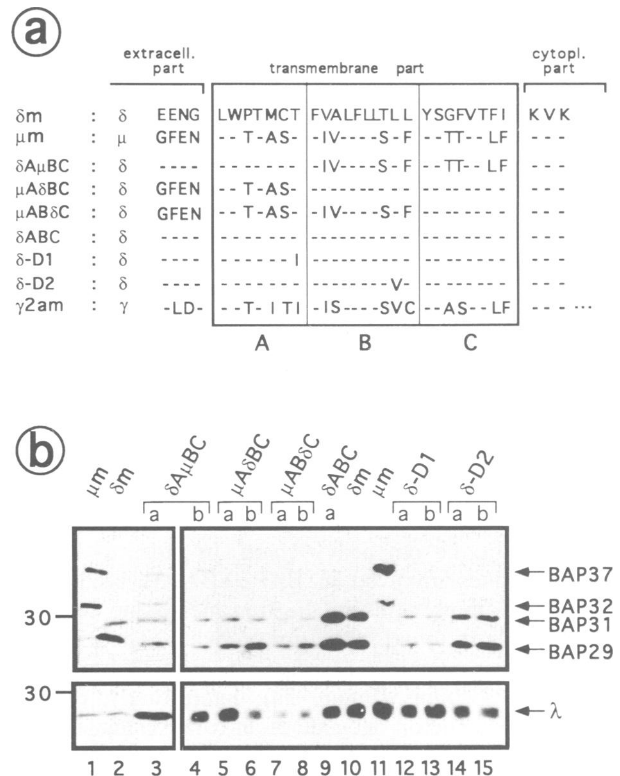


Fig. 5. Analysis of transmembrane sequence mutants of mIgD for BAP binding. (**a**) Comparison of the transmembrane region sequence of wild-type mouse mIgD with that of chimeric and mutated mIgD molecules and that of wild-type mIgM and mIgG2a molecules. The transmembrane region of a mIg molecule consists of an extracellular linker and transmembrane and cytoplasmic sequences. The transmembrane sequence of the mIgD and mIgM molecules was divided into three parts (A, B and C) which were exchanged in different combinations between these two heavy chain classes. Amino acid differences are indicated by a letter and identity with the mouse mIgD wild-type sequence is indicated by a dash. (**b**) Co-purification of BAP29/BAP31and BAP32/BAP37 with wild-type and mutated mIgD as well as with wild-type mIgM. The mIg transfectants of J558L/mb-1 (2×10^6 cells/lane) were lysed in lysis buffer containing 1% Triton X-100 and immunoprecipitated with NP-Sepharose 4B. The affinity-purified proteins were size separated by reducing 10% SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was developed either with a mixture of antisera raised against BAP29, BAP31 and BAP37 and the monoclonal antibody MAK32 (upper panel) or a rabbit anti-mouse λ1 light chain antiserum (lower panel). Protein bands were visualized with the ECL system using a mixture of a HRP-conjugated goat anti-rabbit IgG antibody and a HRP-conjugated sheep anti-mouse IgG antibody.

Isolation and nucleotide analysis of human BAP31 cDNA

We next analyzed the evolutionary conservation of *BAP31* by cloning and sequencing the human *BAP31* cDNA. Using a 600 bp *Eco*RI–*Mrs*I fragment from the mouse *BAP31* cDNA as a probe we screened a total of 8×10^5 phages from human cDNA libraries derived from either HL60 or the pro-B cell line FLEB14-14. Two overlapping positive phage clones were isolated. The insert sequences of these clones revealed an open reading frame of 738 bp begining with an ATG codon which fulfills the Kozak criteria for a good translational start (Figure 1). The open reading frame codes for a protein of 246 amino acids with a strong sequence identity to murine BAP31 protein. The human *BAP31* cDNA and protein are 91 and 95% identical to their mouse counterparts respectively.

A sequence almost identical to the human *BAP31* cDNA was found in the EMBL Database (accession No. X81109). This sequence is derived from the *CDM* gene (Mosser *et al.*, 1994), which is located next to the adrenoleukodystophy gene (Sarde *et al.*, 1994) in the human Xq28 chromosomal region. Thus the mouse and human *BAP31* genes have an evolutinarily conserved location on the X chromosome.

Western blot analysis of BAP31 proteins

A rabbit anti-BAP31 antiserum was raised against a glutathione *S*-transferase (GST)–BAP31 fusion protein carrying 125 amino acids from the C-terminal sequence of mouse BAP31. On a Western blot of lysates from

mouse myeloma J558L and human Burkit lymphoma BL18 cells this antiserum detects proteins of 31 and 30 kDa respectively (Figure 4, lanes 3 and 4). The anti-BAP31 antiserum is specific, as a pre-clearing of this antiserum with GST–BAP31-coupled Sepharose beads prevents detection of the BAP31 proteins (Figure 4, lanes 1 and 2). This analysis shows that the human BAP31 protein migrates faster than mouse BAP31, although its predicted molecular weight is slightly higher than that of the mouse protein.

Mutational analysis of the mIgD–BAP association

A previous analysis (Kim *et al.*, 1994) of chimeric γ2a/δm molecules expressed in J558L cells showed that specific binding to the BAP29/BAP31 pair requires only the sequence encoded by the two 'membrane exons' of the δ gene. This sequence comprises the extracellular linker and the transmembrane and the short cytoplasmic sequences of the δm heavy chain. In order to identify single residues involved in BAP29/BAP31 binding we exchanged amino acids in the transmembrane sequence of δm for those found in the μm sequence. The 25 amino acid long transmembrane part of δm differs from that of μm in 11 positions (Figure 5a). Interestingly, most of these positions lie on one side of an α-helix, the structure most likely adopted by the transmembrane sequence of mIg molecules to cross the lipid bilayer.

Oligonucleotide pairs corresponding to three segments (A, B and C) of the transmembrane sequence of δm and μm were ligated in different combinations into the δm expression vector pSVdmXh. The protein sequence encoded by these segments is depicted in Figure 5a. We also introduced two point mutations into the δm sequence (D1 and D2) by replacing Thr7 and Leu16 with Ile7 and Val16 respectively. The latter amino acids occur at these positions in the transmembrane sequence of the γ2a heavy chain (Figure 5a), which is not associated with any of the BAPs (data not shown).

λ1 light chain and Ig-β-producing J558L cells were co-transfected with expression vectors for the Ig-α protein and the chimeric or mutated δm chains. From each transfection experiment two independent cell clones (a and b) expressing an IgD BCR on their cell surface (data not shown) were chosen for further analysis. The mIgD molecules of each transfectant were affinity purified from a Triton X-100 lysate of 2×10^6 cells, separated by 10% SDS–PAGE and blotted onto nitrocellulose. BAPs co-purified with the diverse mIgD molecules were detected on the Western blot by a mixture of different anti-BAP antisera and the monoclonal anti-BAP32 antibody MAK32. To obtain an internal standard for the amount of purified mIgD molecules we reprobed the blot with an anti-λ1 light chain antibody. The result of the analysis is shown in Figure 5b and summarized in Table I.

As shown previously, wild-type mIgM and mIgD molecules are predominantly associated with the BAP32/BAP37 and BAP29/BAP31 pairs respectively (Figure 5b, lanes 1 and 11 and 2 and 10). In comparison with wild-type mIgD, the chimeric mIgD molecules with a D1 (Thr7→Ile) point mutation showed a 5-fold reduced association with BAP29/BAP31 (Table I and Figure 5b, lanes 12 and 13). This suggests that Thr7 of the δm transmembrane sequence is a contact residue for binding to BAP29/BAP31. In contrast, the D2 (Leu16→Val) mutation had only a small effect on binding to BAPs (Figure 5b, lanes 14 and 15).

All μm/δm chimeric mIgD molecules showed reduced BAP29/BAP31 binding (Figure 5b, lanes 3–8). The C-terminal part of the transmembrane sequence of the δm heavy chain seems to be particularly important for determining the specificity of binding to the BAPs. This is indicated by the fact that chimeric mIgD molecules with a δAμBC heavy chain show a 10-fold reduction in binding to BAP29/BAP31 but are now able to bind the otherwise μm-specific BAP32/BAP37 pair, albeit at a reduced (7%) level (Figure 5b, lanes 3 and 4). The first portion (A) of the δm transmembrane sequence, however, is also an important BAP interaction site, since the μAδBC heavy chain shows reduced BAP binding (Figure 5b, lanes 5 and 6).

Discussion

We have used peptide sequences derived from purified BAP31 protein to clone mouse and human *BAP31* cDNAs which encode proteins of 245 and 246 amino acids respectively. The mouse and human BAP31 proteins are 95% identical, indicating that BAP31 is an evolutionarily conserved protein. BAP31 is 47% identical to the previously identified BAP29 protein. Interestingly, a similar degree of identity (43%) also exists between BAP32 (prohibitin) and BAP37, the two mIgM-associated BAPs, which are <15% identical to the mIgD-associated BAPs. In spite of their sequence differences, the BAPs share similar structural features, as they all display a hydrophobic N-terminus and a charged C-terminus with a predicted α-helical structure. The mouse and human *BAP31* genes are both located on an isogenic region of the X chromosome and map to the *DXMit8* and Xq26–28 region respectively. So far no human disease gene has been mapped to the *BAP31* region.

The three stretches of hydrophobic amino acids at the N-terminus of the BAP31 protein may be transmembrane sequences (Tm-1–Tm-3 in Figure 6). This interpretation is in agreement with a biochemical analysis demonstrating that BAP31 and BAP29 are integral membrane proteins (K.-M.Kim *et al.*, unpublished results). The first hydrophobic stretch could also be a leader sequence, but the finding that an N-terminal sequence of the BAP31 protein matches the beginning of the coding sequence of *BAP31* cDNA argues against this. Thus one structural prediction for BAP31 is that of a membrane protein consisting of three N-terminal transmembrane parts connected by short linker and a long α-helical C-terminus situated in the cytoplasm. Interestingly, the cytoplasmic sequences of BAP31 and BAP29 carry a leucine zipper-like repeat consisting of a stretch of three to five leucine or methionine residues (Figure 6, gray boxes) separated from each other by six amino acids. Leucine zippers have an α-helical structure and are involved in the homo- or heterodimerization of proteins. A biochemical analysis suggests that BAP31 can form a homodimer as well as a heterodimer with BAP29 (K.-M.Kim *et al.*, unpublished results). The observation that similar amounts of BAP31 and BAP29 were always co-precipitated together with mIgD suggests that the mIgD molecule binds only to the heterodimer. The leucine zippers of both BAP proteins may mediate

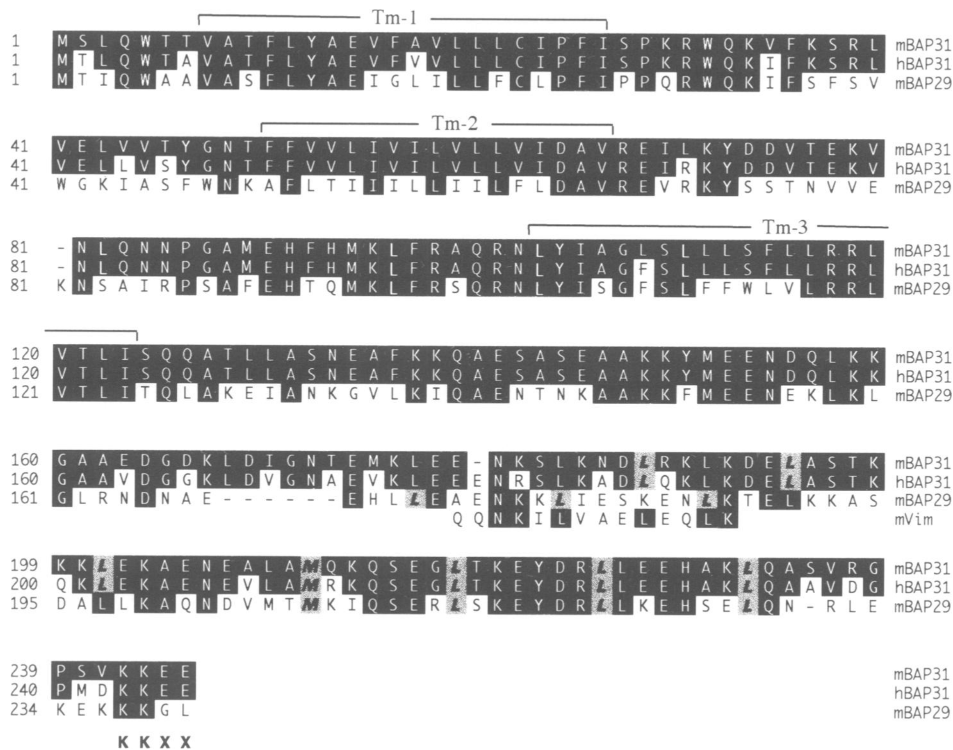


Fig. 6. Comparison of the amino acid sequences of mouse BAP31, human BAP31 and mouse BAP29. Identical amino acids are shown as white letters in black boxes. Amino acids of a leucine zipper-like repeat are indicated by shaded boxes. The putative transmembrane sequences are indicated above the sequence. The KKXX motifs at the C-terminus of BAP31 and BAP29 are indicated by bold below the sequence.

the formation of the BAP dimers. If this is indeed the case, the C-termini of BAP31 and BAP29 would wind around each other in a coiled coil structure. This structural model is supported by the finding that the BAP31 C-terminus dispays a short stretch of sequence homology (Figure 6, amino acids 180–191) to vimentin, an intermediate filament protein with a coiled coil structure.

The function of the BAP31/BAP29 pair is presently unknown. The ubiquitous expression and strong evolutionary conservation of the BAPs suggest that they play an important role in general cellular processes. It has long been known that the cross-linked BCR rapidly associates with the cytoskeleton (Albrecht and Noelle, 1988; Gupta and Woda, 1988), but the elements involved in this association have not been found so far. The BAP31/BAP29 pair may be a candidate for a linker coupling receptors to the cytoskeleton.

Another possible role for the BAPs is control of vesicular transport. After being internalized the antigen–BCR complex can be found in vesicles which are transported to specialized lysosomal compartments where antigen processing takes place (Guagliardi *et al.*, 1990; Liu *et al.*, 1994). Control of these transport processes is still poorly understood. Interestingly, BAP31 and BAP29 both carry a KKXX motif at their C-terminal end. This motif was first described as a retention signal for residual proteins of the endoplasmic reticulum (ER). Recent data suggest, however, that this sequence can also function as a transport signal (Townsley and Pelham, 1994), since it mediates binding to COP proteins, which are involved in the retrograde transport of membrane proteins from the *cis* Golgi to the ER (Letourneur *et al.*, 1994). Furthermore, depending on the last two amino acids, the KKXX motif can also function as an internalization signal (Itin *et al.*, 1995). It is thus possible that the BAP29/BAP31 pair is involved in export or import of receptors to or from the cell surface. In contrast to the mIgD-associated BAPs, the mIgM-associated BAP32/BAP37 pair do not have C-terminal KKXX sequences. However, the latter proteins carry other known internalization signals, like a di-leucine and an NPXY sequence (Terashima *et al.*, 1994). Since they bind to different BAP pairs, the IgM BCR and IgD BCR may have different export or internalization routes.

It has previously been suggested that the conserved and partly hydrophilic transmembrane sequence of mIg molecules not only serves as a membrane anchor, but also as a binding site for other transmembrane proteins (Reth, 1992). Amino acids conserved in all mIg classes lie mostly on one side (Sα/β) of the transmembrane α-helix and are presumably in contact with the Ig-α/Ig-β heterodimer associated with all classes of mIg heavy chain (Venkitaraman *et al.*, 1991). The side opposite (Sbap) to this conserved surface is comprised mostly of amino acids specific for the various mIg classes and could form an interaction surface for the BAPs. Our analysis of transmembrane mutations of the mIgD molecule supports this hypothesis. The D1 point mutation (Thr7→Ile), which reduces binding to the BAP29/BAP31 pair 5-fold, involves a threonine situated on the Sbap side of the transmembrane α-helix. In the chimeric $\delta A\mu BC$ heavy chain five δm-specific amino acids on the lower part of the Sbap side are replaced by μm-specific amino acids, including two threonines and two bulky phenylalanines. In comparison with the wild-type δm chain the chimeric $\delta A\mu BC$ heavy chain binds only 10% of the BAP29/BAP31 pair, but it now shows some (7% in comparison with the wild-

type μm chain) binding to the IgM-specific BAP32/BAP37 pair. Association of mIg molecules with the BAPs therefore seems to be dependent on an array of critical amino acids lying on one side of the transmembrane α-helix. Our data, however, do not exclude the possibility that the extracellular linker sequences of mIgD or mIgM also contribute to BAP binding.

From a broader point of view, our study of BAP–mIg complexes supports the idea that the transmembrane part of membrane proteins can mediate specific protein–protein interactions. The identification of other BAP binding transmembrane proteins may result in a better understanding of the variability and specificity of such interactions in the membrane.

Materials and methods

Cell lines

J558Lδδ/mb-1 (Kim *et al.*, 1994) is a δm/mb-1 co-transfectant of the J558L (H^-, λ^+, $B29^+$, mb-1^-) myeloma cell line. The tumor cell lines WEHI231, K46, Sp6, 33.1.1^-, SCID1, HAFTL4, Ltk^-, BW5147 and MEL1 have been described previously (Terashima *et al.*, 1994). Cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine and 50 mM 2-mercaptoethanol.

Cloning of BAP31 cDNA

According to the amino acid sequence of the two BAP31 peptides K-9 and K-16, the degenerate primers 5′-ATGCA(GA)AA(AG)TA(CT)ATG-GA(AG)GA(AG)AA-3′ and 5′-TT(ACGT)GC(AG)TG(CT)TC(CT)T-C(CT)A(AG)-3′ were synthesized (ABI394/8; Applied Biosystems). These primers were used in a PCR to amplify *BAP31* DNA fragments from cDNA libraries of either the J558L cell line (a kind gift of Drs A.Ehlich and W.Müller, University of Cologne) or of murine spleen and bone marrow cells (Clontech Laboratories Inc.). A 150 bp subfragment of the PCR product was used as a probe to isolate λ phage clones with full-length *BAP31* cDNA inserts from the above indicated libraries. The human cDNA was cloned from a cDNA library of the human pro-B cell line FLEB-14-14 (a kind gift of Drs M.Shirozu and T.Honjo, Kyoto University). The DNA sequence of the inserts was determined by the dideoxy chain termination method (Sanger *et al.*, 1979).

Interspecific backcross mapping

The large backcross panel between the two mouse species *M.spretus* and C57BL/6 containing 982 animals was generated jointly by the Human Genome Mapping Project Resource Centre, UK and the Pasteur Institute, France. F1 females (C57BL/6×*M.spretus*) were backcrossed to C57BL/6 or *M.spretus* males. Each backcross progeny mouse was scored for three to four microsatellite markers per chromosome, thereby completing an anchor map of 70 loci across the mouse genome. A total of 69 backcross mice were used for mapping of the *BAP31* gene.

Southern and Northern blot analyses

Southern blotting analysis of mouse genomic DNA was performed as described (Watanabe *et al.*, 1994). DNA (5 μg) cleaved with various restriction enzymes was separated on a 0.7% agarose gel, transferred to a nylon membrane filter (Hybond N; Amersham) and hybridized with a ^{32}P-labeled *Eco*RI–*Mrs*I 600 bp fragment from *BAP31* cDNA.

Total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). It was then separated on a formaldehyde–agarose gel, transferred to nylon membrane (Biodyne A; Pall) and hybridized with the *BAP31* cDNA probe.

Generation of chimeric and mutated δm transmembrane mutants

First the nucleotides TTAGAG (coding for amino acids 22 and 23 of the M1 exon) were changed by site-directed mutagenesis to CTCGAG, which results in a silent mutation and the introduction of a *Xho*I site into the pSV2δm expression vector. For this purpose the oligonucleotide 5′-TACATGGACCTCGAGGAGGAG-3′ was used. The *Xho*I–*Cla*I fragment encoding the transmembrane region and a part of the M1–M2 intron was subcloned into pBluescript to obtain pBSTM-1. The pBSTM-1 plasmid was cleaved by a *Xho*I/*Stu*I double digestion and the wild-type δm sequence was replaced by three DNA fragments corresponding to the first (A), second (B) and third (C) parts of the transmembrane sequence of either δm or μm. These fragments were ligated in different combinations into the linearized pBSTM-1 plasmid (see Figure 5). For the generation of δm-specific DNA fragments the following oligos were used:
5′-TCGAGGAGGAGAACGGCCTGTGGCCCACAATGTGCAC-3′ (A-upδm) and
3′-CCTCCTCTTGCCGGACACCGGGTGTTACACGTGGAAG-5′ (A-lowδm);
5′-CTTCGTGGCCCTCTTCCTGCTCACACTGCTC-3′ (B-upδm) and
3′-CACCGGGAGAAGGACGAGTGTGACGAGATGT-5′ (B-lowδm);
5′-TACAGTGGCTTCGTCACCTTCATCAAGGTAGG-3′ (C-upδm) and
3′-CACCGAAGCAGTGGAAGTAGTTCCATCC-5′ (C-lowδm).
For the μm-specific DNA fragments the following oligos were used:
5′-TCGAAGGCTTTGAGAACCTGTGGACCACTGCCTCCAC-3′ (A-upμm) and
3′-TCCGAAACTCTTAGACACCTGGTGACGGAGGTGGAAG-5′ (A-lowμm);
5′-CTTCATCGTCCTCTTCCTCCTGAGCCTCTTC-3′ (B-upμm) and
3′-TAGCAGGAGAAGGAGGACTCGGAGAAGATGA-5′ (B-lowμm);
5′-TACAGCACCACCGTCACCCTGTTCAAGGTAGG-3′ (C-upμm) and
3′-CGTGGTGGCAGTGGGACAAGTTCCATCC-5′ (C-lowμm).
The point mutations δm, D1 and D2 were introduced by site-directed mutagenesis with the oligos 5′-ACAATGTGCATCTTCGTGGC-3′ and 5′-CTGCTCACAGTACTCTACAGT-3′ respectively. All mutations and ligations were confirmed by sequencing. The mutated or chimeric transmembrane sequences were isolated as *Xho*I–*Cla*I fragments and cloned into the expression vector pSV2δm.

For each transfection of J558L 10 μg of the the different pSV2δm plasmids and 10 μg of the mb-1 plasmid were used.

Antibodies and antisera

FITC-labeled rat anti-mouse IgD was obtained from Nordic (The Netherlands). Rabbit anti-BAP29, anti-BAP31, anti-BAP32 and anti-BAP37 sera were raised against GST fusion proteins carrying the the C-terminal half of the BAPs. From mice immunized with the same GST fusion proteins we obtained the monoclonal anti-BAP32 antibody MAK32. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG and goat anti-mouse λ were obtained from Southern Biotechnology Associates Inc. (USA).

Precipitation and Western blotting

Cells (2×10^6) were washed in phosphate-buffered saline (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl) and lysed in 0.1 ml lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 10 mg/ml leupeptin, 10 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). After a 10 min incubation insoluble material was removed by centrifugation at 10 000 *g* for 10 min at 4°C. After pre-clearing of the lysate with either Sepharose 4B beads or GST-coupled beads the mIg molecules were precipitated from the supernatant using anti-BAP or NIP-coupled Sepharose 4B beads. The beads were then boiled and the protein in the supernatant size separated by reducing 10% SDS–PAGE. The proteins were electrophoretically transferred to Hybond™ ECL nitrocellulose (Amersham) using as transfer buffer 20 mM Tris-HCl, 150 mM glycine, 20% methanol. Following a 1.5 h transfer the membranes were blocked in 5% skimmed milk and incubated with anti-BAP29, anti-BAP31 and anti-BAP37 antisera and the monoclonal antibody MAK32. After a 2 h incubation the blots were developed with the ECL system (Amersham) using a HRP-conjugated goat anti-rabbit IgG antibody and a HRP-conjugated sheep anti-mouse IgG antibody.

Quantitation of Western blot bands

The Western blot films were digitalized with a video camera, making sure that all exposures were within the linear range. Each band was surface integrated using the CAM software program (Cybertech, Berlin, Germany). The corresponding values were normalized according to the signal of the λ light chain of the BCR and are expressed relative to the mean values for BAP29/BAP31 and BAP32/BAP37 associated with mIgD and mIgM respectively, which were defined as 100%.

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