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The IgM antigen receptor of B lymphocytes is associated with prohibitin and a prohibitin-related protein

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The two major classes of antigen receptors on murine B lymphocytes, mIgM and mIgD, are both contained in a complex with two additional molecules, Ig-α and Ig-β, which permit signal transduction. Accordingly, early biochemical events after antigen binding to either receptor are similar; biological effects, however, are different. Here, we describe three newly discovered intracellular proteins of 32, 37 and 41 kDa molecular mass, that are non-covalently associated with mIgM, but not with mIgD. These proteins coprecipitate with mIgM in Triton X-100 and Nonidet P-40, but not in digitonin lysates. In addition, mIgM is to some extent associated with 29 and 31 kDa proteins that are predominantly associated with mIgD (see accompanying paper). Amino acid sequencing of p32 and p37 identified p32 as mouse prohibitin; this was corroborated by Western blot analysis with antibodies specific for rat prohibitin. p37 is a newly discovered protein. cDNA clones for both proteins were isolated and sequenced. The deduced amino acid sequence of p32 is identical to that of rat prohibitin. p37 is highly homologous to p32. Since prohibitin was identified as an inhibitor of cell proliferation, its association with mIgM, but not mIgD, could explain the different biological events elicited after engagement of each receptor.

Key words: growth control/IgD/IgM/membrane protein/prohibitin

Introduction

Immunoglobulins (Igs) exist in two forms: a secreted and a membrane-bound form (Alt *et al.*, 1980; Early *et al.*, 1980; Rogers *et al.*, 1980). The membrane-bound form (mIg) serves as the antigen receptor on B lymphocytes. In the mouse, IgM and IgD are the most frequently found antigen receptors (Abney and Parkhouse, 1974; Melcher *et al.*, 1974; Vitetta and Uhr, 1975).

Engagement with antigen or other ligands such as anti-Ig antibodies that results in cross-linking of the receptors leads to a cascade of biochemical changes that initiate gene transcription, anchorage of the receptor to the cytoskeleton, receptor endocytosis, antigen processing, antigen presentation, differentiation and cellular proliferation [for reviews see Reth (1992), Cambier *et al.* (1993), Cushley and Harnett (1993), Sakaguchi *et al.* (1993) and Gold and DeFranco (1994)]. mIgM or mIgD by themselves, however, are not sufficient for these effects. Their intracellular tail consists of only three amino acids (Lys, Val, Lys) (Cheng *et al.*, 1982). Therefore, mIgM and mIgD are seconded by a set of disulfide-linked accessory molecules, Ig-α and Ig-β, products of the *mb-1* and *B29* genes, respectively. Ig-α and Ig-β associate as a heterodimer non-covalently with mIg via their membrane-proximal constant domains and transmembrane regions and they facilitate transport of mIgM from the endoplasmic reticulum to the cell surface. Together, mIg and Ig-α and Ig-β form the functional B cell receptor complex (BCR; for literature detailing initial studies on the BCR, see the above-mentioned reviews) which, upon cross-linking, can interact with a set of protein tyrosine kinases and precipitate the intracellular events that lead to activation and differentiation of the B cell. The intracellular domains of Ig-α and Ig-β play an essential role in this process (Clark *et al.*, 1992; Kim *et al.*, 1993b; Law *et al.*, 1993; Matsuo *et al.*, 1993; Sanchez *et al.*, 1993; Flaswinkel and Reth, 1994; Williams *et al.*, 1994). They contain a motif that is found in other intracellular receptor domains and that is fundamental for efficient signal transduction via the antigen receptor (Reth, 1989). It has been referred to as the TAM/ARH1/ARAM motif.

Analysis of the composition of the mIgM– and mIgD–antigen receptor complexes (IgM–BCR and IgD–BCR, respectively) has revealed only limited differences: Ig-α in association with mIgD has a higher molecular mass than that in association with mIgM (33 versus 32 kDa, respectively). This difference is caused by a different glycosylation pattern between Ig-α associated with mIgD or with mIgM. A truncated form of Ig-β, Ig-γ, has also been described. In agreement with the identical intracellular structure, no major differences were found between the two receptor complexes with respect to the induction of early activation events (Pure and Vitetta, 1980a; Cambier *et al.*, 1987; Klaus *et al.*, 1987; Harnett *et al.*, 1989). Experiments designed to reveal functional differences have given contradictory answers: some reports failed to reveal a separate function for either receptor (Layton *et al.*, 1979a,b; Metcalf *et al.*, 1983; Brink *et al.*, 1992). However, numerous reports have described different biological effects following the engagement of mIgM or mIgD *in vivo*, ranging from a role in the induction of antibody responses (Cambier *et al.*, 1978; Pure and Vitetta, 1980b), immunological memory (Zan-Bar *et al.*, 1979) and tolerance (Cambier *et al.*, 1977; Scott, 1978; Zan-Bar and Barzilay, 1982). *In vitro* studies

with splenic B cells (Gaur *et al.*, 1993) or the B cell lymphoma lines WEHI231 (Tisch *et al.*, 1988) and CH31/33 (Ales Martinez *et al.*, 1988), showed that signals generated by cross-linking of mIgM led to programmed cell death (apoptosis), while cross-linking of mIgD failed to do so. Similar results were obtained with human peripheral blood B cells and B cell lymphoma lines (Mongini *et al.*, 1989; Kim *et al.*, 1991, 1992). Furthermore, Carsetti *et al.* (1993) have recently described that engagement of mIgM alone or mIgM and mIgD leads to different activation scenarios *in vivo*: engagement of mIgM alone, in the absence of T cell help, invariably was an inhibitory, apoptotic signal for the B cell, while engagement of mIgM and mIgD together did not lead to apoptosis. Analysis of IgD-deficient mice (Nitschke *et al.*, 1993; Roes and Rajewsky, 1993) confirmed that the absence of IgD reduces the efficacy with which the B cell participates in immune responses.

These observations suggested that signal transduction from the mIgD antigen receptor differed from that from the mIgM receptor. We therefore analyzed the receptor complex in more detail in WEHI231 and splenic B cells. Using a strategy that allowed the identification of proteins that are intracellularly associated with mIgM or mIgD, we describe here three additional proteins of 32, 37 and 41 kDa, that are associated with the mIgM antigen receptor complex, but not with the mIgD complex. In contrast, mIgD and, to a lesser extent, mIgM, are associated with newly discovered 29 and 31 kDa proteins (see accompanying paper by Kim *et al.*). Amino acid sequence analysis of the 32 and 37 kDa proteins identified the 32 kDa protein as prohibitin, a recently described inhibitor of cell proliferation and, possibly, a tumor suppressor gene product (Nuell *et al.*, 1991). Western blot analysis with prohibitin-specific antibodies confirmed the identity with prohibitin. The second 37 kDa protein is the product of an unknown gene. Protein sequence analysis showed that it is strongly related to p32/prohibitin.

Results

Identification of three molecules associated with IgM

Ig-α and Ig-β are easily detected as surface proteins that are associated with the antigen receptor on B lymphocytes in lysates made with the relatively mild detergent digitonin. This is demonstrated in Figure 1 using the B cell lymphoma WEHI231. Surface proteins of WEHI231 cells were labeled with biotin before lysis in 1% digitonin and the receptor complex was precipitated with anti-IgM antibodies and protein G coupled to Sepharose 4B. The precipitated material was separated by reducing SDS–PAGE and transferred to nitrocellulose membranes. Biotinylated proteins were revealed with peroxidase-coupled streptavidin and a luminescent substrate (Figure 1). Broad bands, of 34–39 and 40–55 kDa, corresponding to the heterogeneously glycosylated transmembrane proteins Ig-α and Ig-β respectively, were visible (Kim *et al.*, 1993a), in addition to the bands that represent the components of IgM itself [membrane form of μ (mμ) and κ light chains, respectively] (Figure 1, lane 2) . When cells were lysed in 1% Triton X-100 [or 1% Nonidet P-40 (NP-40), not shown], neither Ig-α, nor Ig-β, nor other proteins that were not IgM itself, were detected (Figure 1, lane 4), demonstrating that the use of stronger detergents disrupts the association between mIgM and Ig-α and Ig-β. However, when cells were first lysed in Triton X-100, followed by the precipitation of IgM with anti-IgM antibodies and protein G–Sepharose and subsequent biotinylation of the precipitated material, bands that were not accounted for by IgM itself or the precipitating antibody were revealed (Figure 1, lane 6): prominent 32 and 37 kDa proteins were visible as well as a faint 41 kDa band. These bands were not seen when lysates were treated with a control (non-immune) antiserum (Figure 1, lane 5). Similar results were obtained using the cell line J558Lμm/mb-1, a plasmacytoma that expresses λ light chains and Ig-β, and, through gene transfer, the membrane form of μ heavy chains and Ig-α (see accompanying paper). The latter data show that the newly identified components of the BCR can associate with mIgM. We called these proteins BAPs (BCR-associated proteins) BAP32, BAP37 and BAP41.

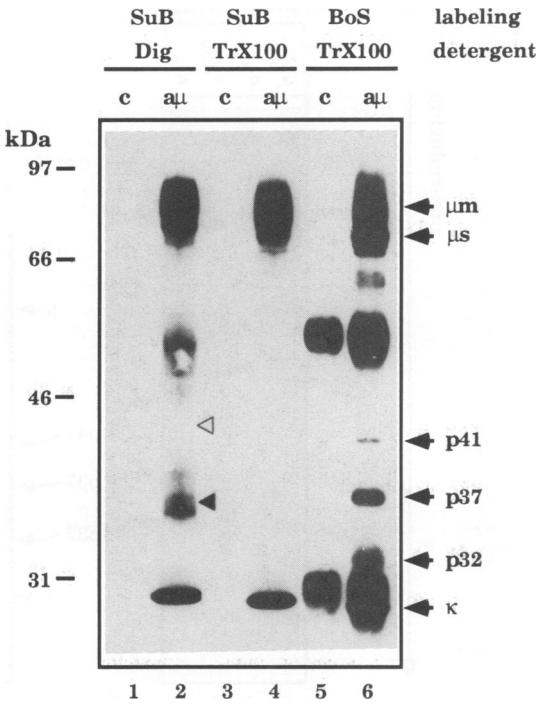

Fig. 1. Analysis of receptor complexes precipitated from digitonin or Triton X-100 lysates of WEHI231 cells. WEHI231 cells (2×10^7 cells/lane) were labeled with biotin before lysis in 1% digitonin (Dig, lanes 1 and 2) or Triton X-100 (TrX100, lanes 3 and 4). Lysates were treated with non-immune control antibody (C, lanes 1 and 3) or goat anti-mouse IgM antibodies and protein G coupled to Sepharose 4B (aμ, lanes 2 and 4)]. Alternatively, the cells were lysed in 1% Triton X-100 followed by precipitation with control antibodies or goat anti-IgM antibodies and protein G coupled to Sepharose 4B (aμ). The washed precipitates were then labeled with biotin (TrX100, lanes 5 and 6). Precipitates were separated by reducing SDS–PAGE, and biotinylated proteins were visualized with HRPO–streptavidin and a luminescent substrate. Bands of specific interest are labeled (▲, Ig-α; △, Ig-β; μm, membrane form of μ; μs, secretory form of μ). SuB: surface biotinylation, BoS: biotinylation on sorbent.

BAP32, BAP37 and BAP41 are non-covalently associated with IgM

We next studied the physical characteristics of the association of IgM with the BAPs. The IgM–BCR of WEHI231 cells was labeled after lysis of the cells and precipitation

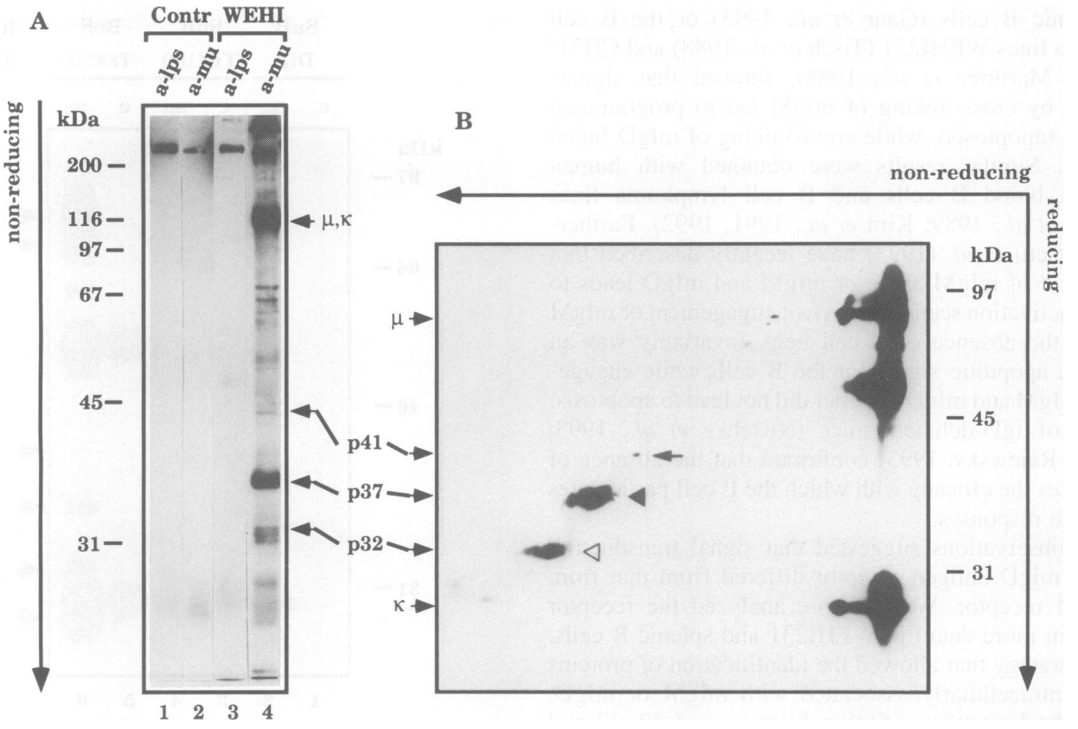

Fig. 2. BAP32 and BAP37 bind non-covalently to IgM. Western blot analysis of receptor complexes from detergent lysates of WEHI231 cells that were biotinylated *after* lysis and precipitation. (**A**) WEHI231 cells (2×10^7 cells/lane) were first lysed in 1% NP-40, lysates were precleared and treated with monoclonal antibodies to LPS (BA1.2b, lane 3) or IgM (b7-6, lane 4), both coupled to Sepharose 4B. Precipitates were labeled with biotin and separated by non-reducing SDS−PAGE, followed by Western blotting; biotinylated proteins were visualized with HRPO−streptavidin and a luminescent substrate. As a control, antibodies coupled to Sepharose 4B, without exposure to lysate, were biotinylated and analyzed as above (lanes 1 and 2). (**B**) WEHI231 cells were lysed in 1% Triton X-100, and the lysates were precleared and treated with goat anti-mouse IgM antibodies and protein G coupled to Sepharose 4B. Precipitates were labeled with biotin, and first separated by non-reducing SDS−PAGE, followed by electrophoresis in the second dimension under reducing conditions. Biotinylated protein was revealed as above.

of the complex. The precipitated material was separated by non-reducing SDS−PAGE and transferred to nitrocellulose membranes. Biotinylated proteins were revealed as before. Results were compatible with those described in Figure 1: IgM was associated with at least three previously unknown molecules of 32, 37 and 41 kDa molecular mass (Figure 2A). These proteins were non-covalently associated with IgM. Control antibodies (anti-LPS) failed to precipitate these bands (Figure 2A). Two-dimensional PAGE then confirmed that these proteins were monomeric and did not form covalently linked multimers (Figure 2B).

Although the experiments do not exclude the possibility that IgM associates with these proteins after detergent lysis, we interpret the data to mean that IgM in WEHI231 cells is non-covalently associated with at least three newly discovered intracellular proteins. Treatment of the precipitated complexes with endoglycosidases did not change the apparent molecular mass of the 32 and 37 kDa proteins (not shown). The proteins are different from the previously described Mb-1 and B29 gene products in that (i) their association is stable in Triton X-100 and NP-40 lysates, (ii) they cannot be biotinylated on the cell surface, (iii) they are not a covalently associated heterodimer and (iv) they are not recognized by anti-B29 monoclonal antibodies (not shown).

BAP32 and BAP37 are associated with IgM, but not IgD on splenic B cells

We then asked whether the antigen receptor complex of splenic B cells was similar to that of lymphoma and myeloma cells. T cell depleted spleen cells were lysed with 1% NP-40 and the receptor complex was precipitated with monoclonal anti-IgM or anti-IgD antibodies coupled to Sepharose 4B. The precipitated material, separated by non-reducing SDS−PAGE and revealed by silver staining, was compared with similarly treated lysates of WEHI231 cells (Figure 3). Two prominent bands at 32 and 37 kDa and a fainter band at 41 kDa were seen in the precipitates obtained with anti-IgM antibodies (lanes 3 and 6). These bands were not visible in the precipitates obtained with anti-IgD antibodies or control (anti-LPS) antibodies (lanes 2 and 5). Both anti-IgM and anti-IgD coprecipitated bands at 29 and 31 kDa (compare lanes 2 and 5 with lane 8, and lanes 3 and 6 with lane 9). The coprecipitation of 29 and 31 kDa proteins with anti-IgD antibodies from lysates of WEHI231 cells was not unexpected because WEHI231 cells express small amounts of IgD [faintly visible in lane 5, see also Haggerty *et al.* (1993)]. From these experiments we conclude that BAP32, BAP37 and possibly BAP41 are intracellular proteins that associate with IgM, but not with IgD. Since the BAP29 and BAP31 are described and discussed in detail in the accompanying paper (Kim *et al.*, 1994) we focus here on the IgM-associated BAPs.

Identification of BAP32 as the product of the prohibitin gene, and BAP37 as an unknown protein related to BAP32/prohibitin

We purified the 32 and 37 kDa proteins from WEHI231 cells to obtain peptide sequences. Two internal sequences

were obtained from BAP32 (Table I). Both independent sequences matched completely with the published sequence of rat prohibitin. Rat prohibitin is a recently described, ubiquitous intracellular protein that has been implicated in the control of cell proliferation and may have tumor suppressor activity (Nuell *et al.*, 1991).

Seven internal sequences were obtained from BAP37 protein (Table I). These peptides showed variable similarity to the rat prohibitin protein, ranging from 80% identity to no similarity. Similar peptide sequence data were obtained from 32 and 37 kDa proteins that were copurified with mIgM from the J558Lμm/mb-1 myeloma cells (Table I).

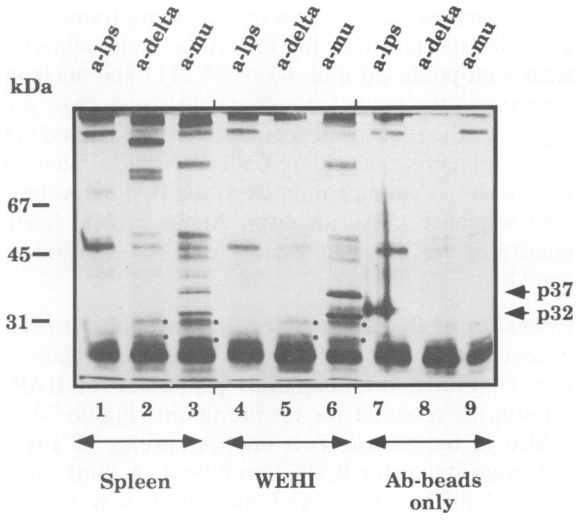

Fig. 3. BAP32 and BAP37 are associated with IgM, but not IgD on splenic B cells. Analysis of receptor complexes precipitated from NP-40 lysates of splenic B cells (lanes 1–3) and WEHI231 cells (lanes 4–6). C57BL/6 splenic B cells (1.3×10^8 cells/lane) or WEHI231 cells (2×10^7 cells/lane) were lysed in 1% NP-40. The soluble fraction was pre-cleared and treated with anti-IgM mAb b7-6 (lanes 3 and 6), anti-IgD mAb 11-26C (lanes 2 and 5), or anti-LPS mAb BA1.2b (lanes 1 and 4), all coupled to Sepharose 4B. Precipitates were separated by non-reducing SDS–PAGE, and proteins were visualized by silver staining. Lanes 7–9 represent controls, where antibody-coupled beads were analyzed without exposure to lysates. Arrows represent BAP32 and BAP37; BAP29 and BAP31 are indicated by dots.

Western blot analysis with prohibitin-specific antibodies

A rabbit anti-prohibitin antiserum, generated against prohibitin-derived peptides (kindly provided by Dr Keith McClung), was used to analyze proteins that coprecipitated with IgM from splenic B cells and WEHI231 cells. Precipitates were analyzed by SDS–PAGE and directly visualized by Coomassie Brilliant Blue staining (Figure 4A) or after Western blot analysis with anti-prohibitin antibodies (Figure 4B). Figure 4A shows proteins corresponding to 32 kDa and 37 kDa molecular mass in anti-IgM precipitates from WEHI231 cells, and faintly in anti-IgM, but not at all in anti-IgD precipitates from splenic B cells. We confirmed the identity of BAP32 with prohibitin (Figure 4B), and corroborated our observation that BAP32/prohibitin is associated with IgM, but not with IgD. Anti-prohibitin antibodies cross-react only minimally with BAP37 (visible on longer exposures, not shown).

Specificity of the interaction of BAP32/prohibitin with IgM

BAP32/prohibitin and BAP37 are associated with IgM in splenic B cells and lymphoma cells. Next, we studied the association of BAP32 and IgM in pre-B cell lines (Figure 5A). We used the cell line $33.1.1^+$, an Abelson-transformed pre-B cell line derived from the Sp6-transgenic mouse expressing μ and κ transgenes, and the transgene-negative variant $33.1.1^-$. The latter cell line was transfected with expression vectors coding for a wild type μ heavy chain or a variant μ heavy chain, in which the membrane-proximal spacer and transmembrane region of μ was replaced with that of MHC class I (Iglesias *et al.*, 1993). They are used here to study the specificity of the interaction of BAP32/prohibitin and IgM. Cell line μ3 expresses the wild type μ heavy chain (secreted and membrane form), cell line μtmH2 expresses the variant μ heavy chain. Both wild type and variant chains are expressed on the cell surface with the pseudo-light chains V_{preB} and λ5 and can be easily biotinylated on the surface (Figure 5A, upper panel, and data not shown). Western blot analysis (Figure 5A, lower panel) shows that BAP32/prohibitin is coprecipitated with IgM in cell lines expressing μ chains with the wild type transmembrane region of μ [$33.1.1^+$(μ,κ) and $33.1.1^-$μ3] but not in those

Table I. p32- and p37-derived peptides

BAP	Source	
	WEHI231	J558μm/mb-1
BAP32/prohibitin	EFTEAVEAK	
	QVAQQEAERARFVVEK	QVAQQEAERARFV
		AAELIANSLATAG
BAP37	LAGRLPAGPR	DLAGRLPAGPRGMGT
	FNASQLI-QRAQVS	NASQLITQRAQVSLLIRRELT
	QVAQQEAQRAQFLVEK	
	IVQAEGEF[a]	
	MLGEALSK	
	IATSQNRIYLTADNLVLNLQD	
	FGLILDDVAS[b]	

[a]Sequence disparity with translated nucleotide sequence.
[b]Poor sequence.

expressing μ chains with the variant transmembrane region of class I molecules. Whereas the total amount of μ protein in lysates of cell lines 33.1.1$^+$(μ,κ) and 33.1.1$^-$μ3 is similar, the expression of μ on the surface of cell line 33.1.1$^+$(μ,κ) is about seven times higher than on the surface of cell line 33.1.1$^-$μ3 [as determined by flow cytometry (Iglesias *et al.*, 1991)]. This result could explain the difference in the amount of copurified BAP32/prohibitin between the two cell lines. These data, and those that show the association of p32 with the membrane form of IgM (see also accompanying paper), indicate that BAP32/prohibitin associates with the transmembrane region and/or membrane-proximal spacer of the IgM molecule and suggest an association of BAP32 with surface-bound IgM.

Further evidence for the specificity of the interaction of BAP32/prohibitin and mIgM was obtained from analysis of other membrane proteins on the B lymphoma line CHB3.LX. CHB3.LX expresses, among others, the membrane-bound receptors CD25 (α chain of interleukin-2 receptor), CD32 (FcγRII) and CD44 (hyaluronate receptor) and a limited amount of mIgD. After surface biotinylation and precipitation with specific antibodies, these molecules and IgM are revealed on a Western blot with horseradish peroxidase (HRPO)-conjugated streptavidin and a luminescent substrate (Figure 5B, upper panel). BAP32/prohibitin associated exclusively with IgM, but not with the other tested membrane proteins (Figure 5B, lower panel).

We conclude that BAP32 (and BAP37) are intracellular proteins that specifically interact with the transmembrane domain of IgM in cells of the B lineage, but not with that of some other surface molecules including mIgD.

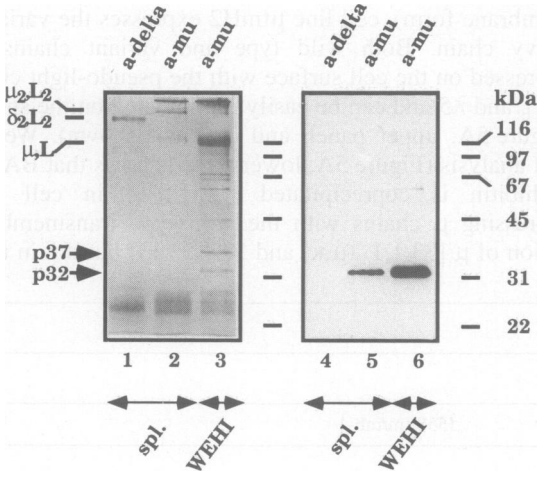

Fig. 4. Identification of BAP32 as mouse prohibitin. C57BL/6 splenic B cells (lanes 1, 2, 4 and 5, 1.3×10^8 cells/lane) or WEHI231 cells (lanes 3 and 6, 5×10^7 cells/lane) were lysed in 1% NP-40. The soluble fraction was pre-cleared, followed by treatment with an anti-IgM mAb b7-6 (lanes 2, 3, 5 and 6) or an anti-IgD mAb 11-26C (lanes 1 and 4), both coupled to Sepharose 4B. Precipitates were separated by non-reducing SDS–PAGE, and proteins were blotted onto nitrocellulose membranes and visualized by Coomassie Brilliant Blue staining (left panel). The blot was then incubated with anti-prohibitin antiserum, and developed with HRPO-labeled goat anti-rabbit antibody and a luminescent substrate (right panel). The mobility of BAP32 and BAP37 is indicated, as is the mobility of mIgM (μ_2L_2), mIgD (δ_2L_2) and mIgM hemimers (μL).

Cloning of the mouse cDNA for BAP32/prohibitin and BAP37

Published sequences from the coding region of the rat prohibitin cDNA (Nuell *et al.*, 1991) were used to amplify the corresponding mouse sequences with the PCR technique. The amplified product was used to isolate full-length cDNA clones from a J558L-derived cDNA library. Nucleotide sequence analysis showed that the mouse and rat sequence were 93% identical. The amino acid sequence deduced from the mouse cDNA (Figure 8) showed complete identity with rat prohibitin, and predicts a mol. wt of 29 817. Degenerate primers deduced from the peptide sequences were used to amplify BAP37-specific cDNA sequences. These were used to isolate five positive clones from a pre-B cell cDNA library. The composite nucleotide sequence analysis revealed an open reading frame of 894 nucleotides starting from the first ATG, and coding for a protein with predicted mol. wt of 33,271. The nucleotide sequence and the predicted amino acid sequence is given in Figure 6. The sequenced peptides of BAP37 found in the deduced protein sequence are underlined. The nucleotide sequence shows strong similarity to the BAP32/prohibitin coding sequence (57% identity). At the protein level, a similarity of 72% and an identity of 51% were inferred (Figure 8).

Expression of BAP32/prohibitin and BAP37 mRNA

Northern blot analysis of various mouse cell lines and tissues confirmed the ubiquitous expression of BAP32/prohibitin, as reported for rat prohibitin (Figure 7A and B). Also in the mouse, two mRNA species of 1.6 and 2.0 kb were found for BAP32/prohibitin. A third, shorter species of mRNA for BAP32/prohibitin was found in testis. Similar results were obtained by Choongkittaworn *et al.* (1993). The expression pattern of BAP37 mRNA was similar to that of BAP32/prohibitin; only one band was detected at 1.7 kb (Figure 7A and B). The expression of BAP32/prohibitin and BAP37 mRNAs in cell lines was consistently much stronger than that in tissues.

Discussion

In this paper we have identified three possible new members of the IgM antigen receptor complex of B cells, namely BAP32, BAP37 and BAP41, and characterized two of them. These molecules associate only with mIgM, and not with mIgD. Two more constituents of the IgD antigen receptor complex, BAP29 and BAP31, have also been identified; they are also weakly associated with mIgM and are discussed in detail in the accompanying paper (Kim *et al.*, 1994). BAP32 is identical to rat prohibitin, a recently discovered, ubiquitous, intracellular 32 kDa molecule (Nuell *et al.*, 1991). The second mIgM-associated protein, BAP37, is an unknown protein but is related to BAP32/prohibitin. Like BAP32/prohibitin mRNA, BAP37 mRNA is ubiquitously expressed. BAP41 was not further characterized. The presumed function of BAP32/prohibitin, namely inhibition of cell proliferation, fits well with the reported functional differences between mIgM and mIgD.

A tale of two receptors

The presence of two different antigen receptors with the same specificity on mature B lymphocytes has puzzled

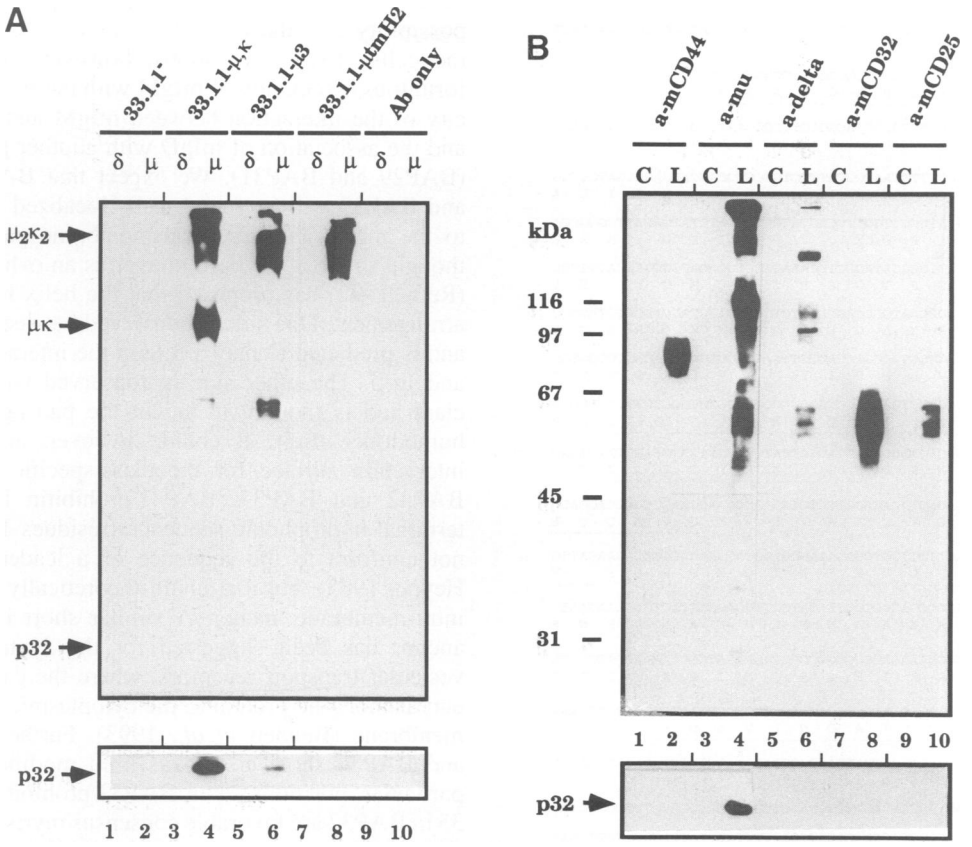

Fig. 5. BAP32/prohibitin specifically associates with the membrane domain of IgM. (**A**) Analysis of AMuLV-transformed pre-B cells. Cell line 33.1.1$^+$ was derived from a μ and κ transgenic mouse line, and expresses mIgM. Cell line 33.1.1$^-$ was an IgM-loss variant of 33.1.1$^+$. Cell line 33.1.1$^-$ was transfected with μ-derived constructs as detailed in the text. Cells (7×10^7 cells/lane) were lysed in 1% NP-40 and the detergent-soluble fraction was treated with either anti-IgM mAb b7-6 (lanes 2, 4, 6, 8 and 10) or, as a control, anti-IgD mAb 11-26C (lanes 1, 3, 5, 7 and 9) followed by protein G–Sepharose. The precipitates were separated by non-reducing SDS–PAGE followed by Western blotting. Blots were revealed with goat anti-mouse IgM (upper panel) or rabbit anti-prohibitin antibody (lower panel) and appropriate HRPO-labeled second antibodies and a luminescent substrate. The mobility of mIgM (μ_2k_2), mIgM hemimer (μk) and BAP32 is indicated. (**B**) Analysis of surface receptors other than IgM on the cell line CHB3.LX. Lanes labeled L: cells (1×10^8 cells/lane) were biotinylated before lysis in 1% Triton X-100, detergent-soluble lysates were pre-cleared, followed by treatment with mAb KM81 (anti-CD44), mAb 29-11 (anti-IgM),mAb AF6-122 (anti-IgD^b), mAb 2.4G2 (anti-CD32) or mAb PC61 (anti-CD25), all coupled to Sepharose 4B. Precipitates were separated by non-reducing SDS–PAGE, followed by Western blotting. Proteins were revealed by HRPO–streptavidin (upper panel) or rabbit anti-prohibitin antibody (lower panel) as above. Lanes labeled C: controls; antibodies, coupled to Sepharose 4B, were biotinylated and treated as above, without exposure to lysates. For CD25 and CD32 a molecular mass of ~55 kDa and for CD44 a molecular mass of ~85 kDa is expected.

immunologists since the discovery of the second receptor, IgD.

mRNA for μ and δ heavy chains arise from the same primary transcript through differential splicing (Maki *et al.*, 1981). Except for an identical antigen-binding domain, murine mIgM and mIgD are rather different in structure. μ heavy chains consist of four constant (C) domains, wheras δ heavy chains possess two C domains separated by a large hinge region (Burton, 1987). The membrane-proximal C domains of IgM and IgD, which show limited similarity, are separated from the transmembrane domains by spacer sequences, which show no obvious similarity. The transmembrane parts are similar (56% identity), while the intracellular tails are identical (Lys, Val, Lys) (Cheng *et al.*, 1982). This last sequence is too short to convey signals after binding of antigen. Recently, a complex of two disulfide-linked molecules, Ig-α and Ig-β, that associates with the membrane-proximal and transmembrane domains of all Ig classes has been described [for detailed references, see reviews by Reth (1992), Cambier *et al.* (1993), Cushley and Harnett (1993), Sakaguchi *et al.* (1993) and Gold and DeFranco (1994)]. It is responsible for signal transduction.

In immature B cells which express solely mIgM (Yuan, 1986), the antigen receptor is required for differentiation and, possibly, for selection into the peripheral immune system (Alt *et al.*, 1987; Reth *et al.*, 1987; Era *et al.*, 1991; Kitamura *et al.*, 1991). Later in development, mature, resting B cells also express mIgD (Abney and Parkhouse, 1974; Melcher *et al.*, 1974; Vitetta *et al.*, 1975), the antigen receptors are then responsible for the recognition of foreign antigens. After binding of antigen and subsequent activation, the mRNA level for δ heavy chain drops and expression of mIgD decreases (Yuan and Tucker, 1984). This developmentally regulated expression and the virtual absence of a secreted form of IgD in serum predicted a role for mIgD in the differentiation and activation of B cells.

Initial experiments failed to reveal a difference between mIgD and mIgM regarding the transduction of signals: both induced early activation events like protein phosphorylation, phosphoinositol turnover, Ca^{2+} fluxes and

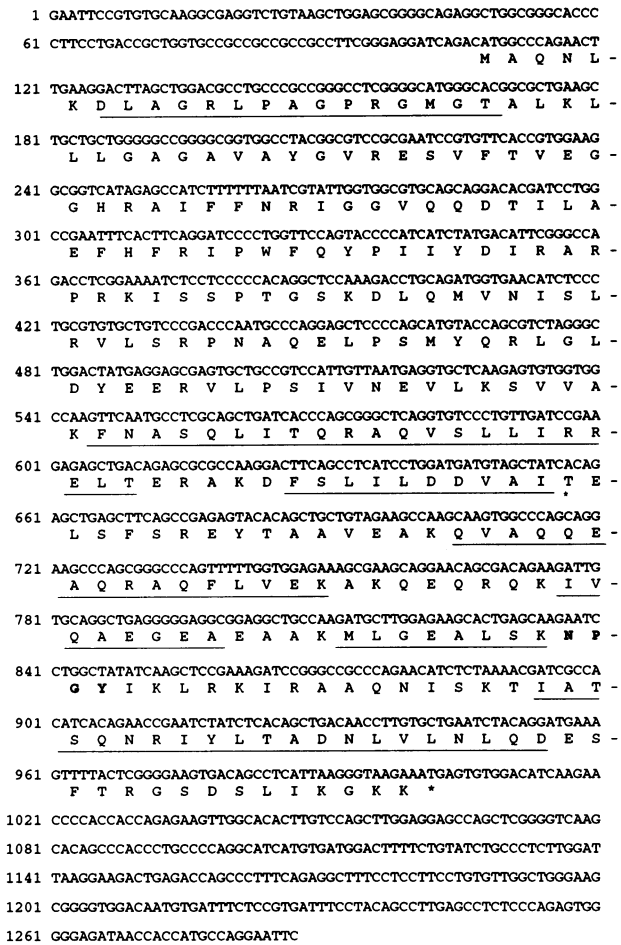

Fig. 6. The nucleotide and predicted amino acid sequences of BAP37. The nucleotide sequence was determined as detailed in Materials and methods. The deduced amino acid sequence is given starting from the first ATG. The single letter amino acid code is used. The sequenced peptides are underlined.

DNA synthesis. Analysis of events over a longer period, however, showed that in the WEHI231 mouse cell line, and in other murine and human cell lines, mIgM conveyed an apoptotic signal, while mIgD failed to elicit this response (Ales Martinez *et al.*, 1988; Tisch *et al.*, 1988; Mongini *et al.*, 1989; Kim *et al.*, 1992). Carsetti *et al.* (1993) have recently extended these studies in a transgenic mouse model and have shown that *in vivo*, mIgM and mIgD exert dramatically different functions: engagement of mIgM alone on mature B cells led to B cell deletion by apoptosis, while engagement of mIgD and mIgM together failed to induce programmed cell death.

These experiments implied that mIgM and mIgD are wired differently to the signaling cascade in the cell. The discovery of a set of proteins that is uniquely associated with mIgM, but not mIgD, could provide a molecular basis for these functional differences.

The association of BAPs with mIgM

The evidence brought forward in this paper suggests that the transmembrane domain of mIgM determines the specificity of the interaction with BAP32/prohibitin and BAP37. The topography and the nature of the interaction of mIgM and BAP32/prohibitin and BAP37 is unknown. As a word of caution, we cannot at present exclude the possibility that the association first occurs after lysis of the cells. Two observations, however, argue against a fortuitous association of mIgM with the BAPs: the specificity of the interaction between mIgM and the BAP pair, and the association of mIgD with another pair of proteins (BAP29 and BAP31). We expect that BAP32/prohibitin and BAP37 will be found to be localized in or proximal to the membrane. The transmembrane part of mIgM is thought to cross the lipid bilayer as an α-helix. One of us (Reth, 1992) has proposed that the helix has a two-sided arrangement. One side is conserved between all Ig classes and is predicted to play a role in the interaction with Ig-α and Ig-β. The other side is conserved within the mIgM class and is thought to aid in the pairing of the mIgM homodimer itself. It could, however, also function as interaction surface for the class-specific association of BAP32 and BAP37. BAP32/prohibitin has an amino-terminal hydrophobic sequence (residues 1–25) that does not conform to the sequence of a leader peptide (Von Heyne, 1985), but that could theoretically function as an intra-membrane anchor. A similar short intra-membrane anchor has been suggested for the syntaxin family of vesicular transport receptors, where the carboxy-terminal end anchors the protein to the cytoplasmic side of the cell membrane (Bennett *et al.*, 1993). Further, both BAP32 and BAP37 show consensus lipid modification sites (in particular, positions 21 in BAP32/prohibitin and position 35 in BAP37 are favorable consensus myristoylation sites). For myristoylation, the amino-terminal end needs to be cleaved off (Grand, 1989). The molecular weight determination, however, virtually excludes extensive modifications of either protein. *In vitro* translation and digestion studies by Nuell *et al.* (1991) have shown that the bulk of the prohibitin protein is in the cytoplasm. An alternative model would predict the presence of another, not yet identified adaptor protein. BAP41 is not a likely candidate because of its low abundance in the IgM–BCR precipitates.

The difference in the amount of BAP32/prohibitin that coprecipitated with IgM in lysates of the cell lines $33.1.1^{+}(\mu,\kappa)$ and $33.1.1^{-}\mu 3$ correlated with the amount of μ expressed on the surface of these cells, suggesting an association of BAP32 with surface-bound IgM. Unfortunately, the anti-prohibitin antiserum is not suitable for precipitation or immunofluorescence studies. The availability of antibodies that can be used in such experimental approaches would help to clarify what the exact cellular localization of the BAPs is, and which other proteins associate with the BAPs.

Further biochemical characteristics of BAP32 and BAP37

Immunoprecipitation studies have shown that in lysates of splenic B cells and WEHI231 cells only a small fraction of the BAPs is bound to IgM. Gel filtration analysis further showed that in lysates of spleen cells the BAPs are predominantly found in complexes of higher molecular mass (~120–200 kDa, unpublished observations). When BAP32/prohibitin and BAP37 were coprecipitated with IgM from lysates of WEHI231 cells or splenic B cells and separated by SDS−PAGE, staining with either Coomassie Brilliant Blue or silver revealed bands of about equal density. Thus, we estimate that BAP32/prohibitin and

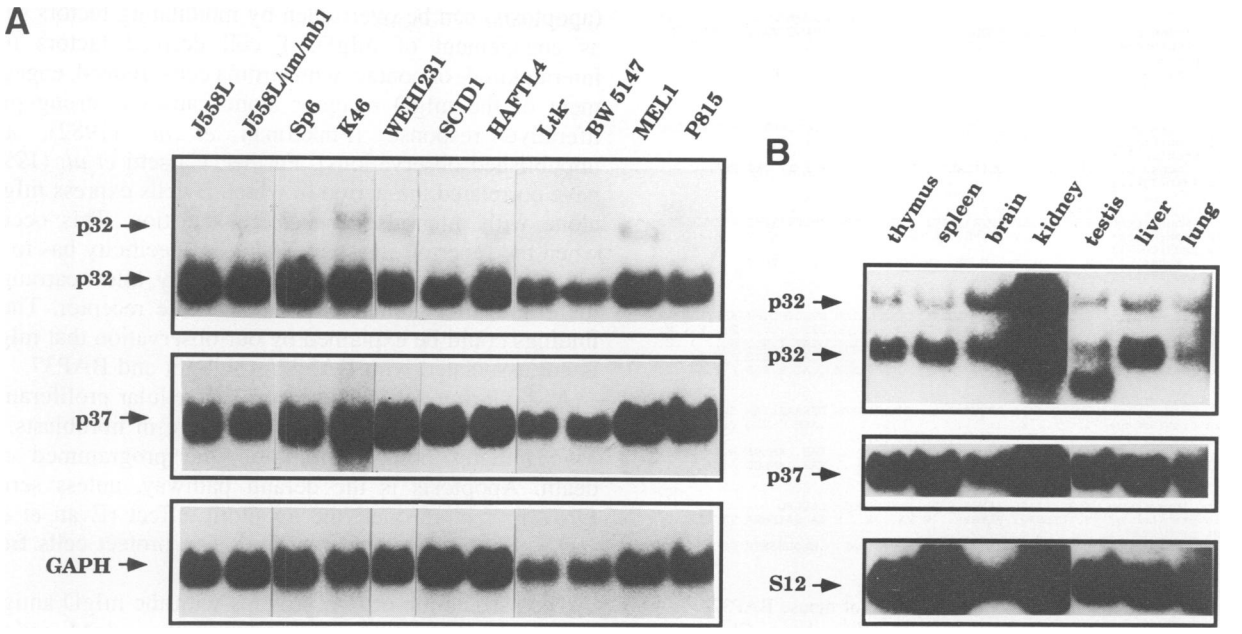

Fig. 7. Northern blot analysis of different mouse cell lines (**A**) and tissues (**B**). Total cellular RNA was isolated from cell lines and tissues, 15 μg RNA was loaded per lane. Blots were consecutively hybridized with randomly primed cDNA fragments of BAP32/prohibitin and BAP37. For comparison, the Northern blot with RNA from cell lines was hybridized with a GAPDH probe, the blot from tissues was standardized with a probe for ribosomal protein S12 mRNA.

BAP37 are present in a 1:1 ratio in IgM precipitates. The almost equimolar association of BAP32 and BAP37 with mIgM suggests that they might form a dimer. However, neither BAP32/prohibitin nor BAP37 possesses a known dimerization motif. We have searched the sequence of BAP32 and BAP37 for other motifs that could give clues as to the nature of the association and to the function of the two proteins. The secondary structure of BAP32/prohibitin and BAP37 as predicted by the Chou–Fasman method (Fasman, 1989) showed a striking similarity in the arrangement of β-sheets and α-helices. Consensus N-linked glycosylation sites, which are apparently not used, are found. Acceptor sites for both serine/threonine and tyrosine kinases are also present. Preliminary experiments, however, failed to show a constitutive or induced phosphorylation of BAP32/prohibitin and BAP37. Furthermore, close to the carboxy-terminus, an NPXY motif is found in BAP37 (Figure 6, bold italic lettering). This motif is frequently found in the cytoplasmic part of transmembrane proteins and is required for the coated pit-mediated internalization of the low density lipoprotein receptor (Chen *et al.*, 1990). Taken together, these observations suggest that these proteins could have a role in the internalization of the IgM–BCR. In B cells, internalization is not only important for the uptake and processing of nominal antigen (Lanzavecchia, 1990), it could also abrogate signals transduced by the receptor by removal of the receptor from the surface.

BAP32/prohibitin is a conserved protein

mRNA for BAP32/prohibitin and BAP37 is ubiquitously expressed. BAP32/prohibitin is found in all cell lines and tissues tested [unpublished observations; see also Nuell *et al.* (1991) and Choongkittaworn *et al.* (1993)]. This implies that their function is not limited to accessory proteins for mIgM, but that they presumably also interact with other receptors. BAP32/prohibitin is a highly conserved gene. Homologs have been found in yeast (Franklin and Jazwinski, 1993), nematodes, *Drosophila* [*Cc* gene (Eveleth and Marsh, 1986)], man (White *et al.*, 1991; Sato *et al.*, 1992) and rat (Nuell *et al.*, 1991). In yeast, the prohibitin homolog has been reported to increase the life span of the cell (Franklin and Jazwinski, 1993). The function of the *Drosophila* Cc gene product is unknown, but it is contained in a lethal complementation group and seems to be required for the transition from larva to pupa (Eveleth and Marsh, 1986). In rat, prohibitin was isolated as a gene expressed in quiescent cells, and it could interfere with cellular proliferation (Nuell *et al.*, 1991).

A search of the GenBank database revealed a cDNA of unknown function derived from human muscle that was very similar to the BAP37. The protein sequences of BAP32/prohibitin, BAP37 and their homologs are aligned in Figure 8 after correcting several possible errors in the published sequences (see legend to Figure 8). From this comparison it is clear that BAP32 and BAP37 form a conserved gene family. The central cores of the proteins show a particularly high degree of conservation.

Functional considerations

Prohibitin was identified as a gene product with anti-proliferative activity. Although the mechanism of this inhibition is unknown, McClung and colleagues (Roskams *et al.*, 1993) have recently brought forward evidence for a role of prohibitin in the regulation of the progression of the cell cycle. Choongkittaworn *et al.* (1993) have shown that prohibitin is differentially expressed in the testis in a pattern that agrees with an anti-proliferative effect of prohibitin. White *et al.* (1991) hypothesized that prohibitin could be a tumor suppressor. Prohibitin has been mapped to mouse chromosome 11 (Morris *et al.*, 1993), and the homologous human chromosome region 17q21 (White

```
                    1                                                  50
          mouse proh MAAKVFESIG KFGLAL---- ---------- AVAGGVVNSA LYNVDAGHRA
drosophila proh(Dmccg) ...QF.NR.. QM.SEX---- ---------- ..L..... ....EG....
 c-elegans proh(Ce371)
          mouse p37 ..QNLKDLA. RLPAGPRGMG TALKLLLGAG ...Y..RE.V -FT.EG....
  human p37(Hsb13g012)

                   51                                                 100
          mouse proh VIFDRFRGVQ DIVVGEGTHF LIPWVQKPII FDCRSRPRNV PVITGSKDLQ
drosophila proh(Dmccg) ....T...IK EN..... F.....R... .---..Q.... .E.....
 c-elegans proh(Ce371)
          mouse p37 IF.N.IG... QDTILAEF.. R...F.Y... Y.I.A...KI SSP.....
  human p37(Hsb13g012)

                  101                                                 150
          mouse proh NVNITLRILF RPVASQLPRI YTSIGEDYDE RVLPSITTEI LKSVVARFDA
drosophila proh(Dmccg) .........Y ..IPD...K. ..IL.Q.... ......AP.V ..A...Q...
 c-elegans proh(Ce371)                                               RQ...
          mouse p37 M...S..V.S ..N.QE..SM .QRL.L..E. ......VN.V ......K.N.
  human p37(Hsb13g012)

                  151                                                 200
          mouse proh GELITQRELV SRQVSDDLTE RAATFGLILD DVSLTHLTFG KEFTEAVEAK
drosophila proh(Dmccg) ........M. .QR..QE..V ..KQ..F... .I........ R...L...M.
 c-elegans proh(Ce371) H.M.....V. .QRA.V..R. ...Q...L.. .IAI...N.. R.......M.
          mouse p37 SQ.....AQ. .LLIRRE... ..KD.S.... ..AI.E.S.S R.Y.A.....
  human p37(Hsb13g012)                      D.S.X.X ..AI.E.S.S R.Y.A.....

                  201                                                 250
          mouse proh QVAQQEAERA RFVVEKAEQQ KKAAIISAEG DSKAAELIAN SLATAGDGLI
drosophila proh(Dmccg) ........K. .......... .L.S...... .AER.C-VGQ VI.RPETVWW
 c-elegans proh(Ce371) ........K. .YL......M .I..VTT...
          mouse p37 .......Q.. Q.L....K.E QRQK.VQ... EAE..KMLGE A.SK-NP.Y.
  human p37(Hsb13g012) .....X.Q.. X.L....K.E QRQK.VQ... EAE..KMLGE A.XK-XX.Y.

                  251                                                 300
          mouse proh ELRKLEAAED IAYQLSRSRN ITYLPAGQSV LLQLPQ
drosophila proh(Dmccg) S..LIDRPRY RLTSYP...G VA...S...H AA.SAIDHRA VAGCI
 c-elegans proh(Ce371)
          mouse p37 K...IR..QN .SKTIAT.Q. RI..T.DNL. .NLQDESFTR GSDSLIKGKK
  human p37(Hsb13g012) X...IR.DXN XSKXX
```

Fig. 8. Comparison of the amino acid sequence of mouse BAP32/prohibitin, *Drosophila* Cc (Dmccg), *Caenorhabditis elegans* CE371, mouse BAP37 and human BAP37 (Hsb13g012). Identities are indicated by a point, and gaps by a dash. The nucleotide sequence of Cc genomic DNA and mouse prohibitin cDNA were compared. Based on the occurrence of two gaps (following position 325 and 329 of Cc, numbering according to the Dmccg entry in the EMBL database) and two insertions (at positions 481 and 482 of Cc) in the comparison and the optimal alignment of deduced amino acid sequence including reading frame shifts, the sequence of Cc was adapted to obtain an optimal alignment with mouse prohibitin by the introduction and deletion of two nucleotides respectively, at the position where gaps and insertions occurred. The intron–exon boundary was placed at position 712 instead of at position 655 as stated in the annotated EMBL entry of Dmccg. A similar procedure was used to optimally align Hsb13g012 with mouse BAP37: one nucleotide was deleted at position 256 and one added after position 107 in the published entry of the EMBL database. The sequence of mouse BAP32/prohibitin and mouse BAP37 are deposited with the EMBL database under accession numbers X78682 and X78683, respectively.

et al., 1991; Sato *et al.*, 1992). In the latter locus, a gene (or gene cluster) has been identified that is related to the occurrence of familial, early onset breast cancer (Smith *et al.*, 1992). The region is also often deleted in other carcinomas, e.g. ovarian carcinoma, supporting the notion that prohibitin is a tumor suppressor gene product. Recent evidence indicates that prohibitin maps just outside the locus responsible for familial, early onset breast cancer (BRCA locus), and that it may be unmutated in ovarian carcinoma (Cliby *et al.*, 1993).

The high degree of similarity of BAP32/prohibitin and BAP37 suggests a similar function for the two proteins. It will be of interest to determine the chromosomal localization of BAP37.

The capacity of prohibitin to regulate proliferation could have important implications for the function of the mIgM–BCR. After cross-linking of mIgM, it appears that two (conflicting) signals are generated: one involves the activation of Src-related kinases and the ensuing activation of immediate-early response genes, leading to cellular proliferation, while the second signal, possibly mediated through BAP32/prohibitin, initiates programmed cell death. The default pathway appears to be apoptosis and, therefore, successful proliferation requires its suppression. In a 'normal' immune response, the default pathway (apoptosis) can be overridden by modulating factors such as engagement of mIgD, T cell derived factors like interleukin-4, or contact with stroma cells. Indeed, engagement of the mIgD receptor alone causes a strong proliferative response [Finkelman *et al.* (1982), and unpublished observations]. Further, Carsetti *et al.* (1993) have correlated the period in which B cells express mIgM alone with intervals of negative selection. This occurs when the repertoire is shaped and its specificity has to be checked for autoreactivity and effectivity, after rearrangement and after somatic mutation of the receptor. These findings could be explained by our observation that mIgD is not associated with BAP32/prohibitin and BAP37.

A similar model for the control of cellular proliferation was proposed for the activity of c-Myc in fibroblasts: c-Myc initiates both proliferation and programmed cell death. Apoptosis is the default pathway, unless serum (growth) factors quell the apoptotic effect (Evan *et al.*, 1992). Such dual control systems can protect cells from uncontrolled proliferation.

The association of two proteins with the mIgD antigen receptor and, to some extent, with the mIgM antigen receptor (see accompanying paper), could suggest that mIgD more efficiently performs certain functions that are also associated with antigen binding to mIgM, like internalization of antigen and antigen presentation (Lanzavecchia, 1990).

Materials and methods

Culture conditions and cell lines

WEHI231 is a non-secreting B cell lymphoma, induced by mineral oil. CHB3.LX is a B cell lymphoma (Bishop *et al.*, 1993), generously provided by Dr G.Haughton, NC. Besides IgM, it expresses small amounts of IgD of allotype b. Cell line 33.1.1$^+$ was obtained through transformation of bone marrow cells of the Sp6 mouse, expressing μ and κ transgenes, with Abelson murine leukemia virus (AMuLV). Cell line 33.1.1$^-$ is a transgene-negative variant of 33.1.1$^+$ (Iglesias *et al.*, 1993). J558L is a mouse myeloma cell line, expressing only λ light chains, and Ig-β- but not Ig-α-associated proteins (Hombach *et al.*, 1988). J558L/μm/mb-1 is transfected with constructs coding for Ig-α and the membrane form of μ. It expresses mIgM. Sp6 is a mouse hybridoma and secretes IgM. K46 is a mouse B cell lymphoma, expressing IgG2a. SCID 1 is an AMuLV-transformed pre-B cell line derived from a severe combined immunodeficiency (SCID) mouse. HAFTL is a mouse pro-B cell line obtained after transformation of fetal liver cells with AMuLV. BW5147 is a mouse thymoma cell line, and MEL1 a mouse erythrocyte leukemia cell line. P815 is a mouse mastocytoma cell line [for references to these cell lines see Wenger *et al.* (1991)]. All cells were cultured in Iscove's modified DMEM, supplemented with 7.5% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol.

Mice

C57/BL6 mice were obtained from our breeding facility. The mice were housed under SPF conditions, and killed at 8–10 weeks of age. B cells were isolated from spleens. Red blood cells were removed by isotonic lysis, and T cells were removed by treatment with a combination of monoclonal anti-CD4 (GK1.5), anti-CD8 (19-178) and anti-Thy1 (AT83) antibodies and complement.

Antibodies

Goat anti-mouse IgM and goat anti-rabbit IgG (unlabeled or HRPO-labeled) were purchased from Southern Biotechnology Associates. Rabbit anti-prohibitin antibody was a gift of Dr K.McClung, Oklahoma City, OK. MAb 11-26C (anti-δ) was a gift of Dr J.Kearney, Birmingham, AL., mAb anti-LPS (BA1.2b) was a gift of Dr H.Waldmann, Cambridge, UK. Anti-CD44 (KM-81), anti-CD25 (PC61), anti-CD32 (2.4G2), anti-μ (b7.6,29-11 and c2.23) and anti-δ^b (AF6.122) were in our collection or

obtained from the ATCC. Antibodies were harvested from culture supernatants and purified on protein G–Sepharose (Pharmacia). Where indicated, antibodies were coupled to cyanogen-bromide-activated Sepharose 4B (Pharmacia), according to the suggestions of the manufacturer.

Labeling with biotin

Cells were washed three times with Hank's buffered saline (HBSS), incubated with sulfo-NHS-biotin (Pierce) at 50 μg/ml in HBSS for 8 min at room temperature and washed three times with HBSS. Precipitates were washed with lysis buffer (see below) followed by two washes with HBSS, and treated with sulfo-NHS-biotin as above. Precipitates were washed three times after biotinylation with the appropiate lysis buffer.

Cell lysis, immunoprecipitation, SDS–PAGE, staining with silver or Coomassie Brilliant Blue, and Western blotting

Cells were lysed in lysis buffer (50 mM Tris, 137 mM NaCl, pH 7.6) containing protease and phosphatase inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF, 1 mM Na_3VO_4) and detergent (digitonin, NP-40 or Triton X-100, at 1%) for 1 h on ice. The detergent-soluble fraction was obtained by centrifugation for 10 min at 14 000 *g*.

Lysates were precleared with glycine-inactivated Sepharose 4B (Pharmacia) or protein G coupled to Sepharose 4B (Pharmacia) before precipitation, unless otherwise stated, for 2 h on ice, followed by incubation with the specific antibody, coupled to Sepharose 4B. Precipitation was allowed to proceed for 2 h, then precipitates were washed three times with lysis buffer and once with HBSS, and collected after centrifugation.

Reducing and non-reducing SDS–PAGE was performed according to Laemmli (Laemmli, 1970).

Proteins were either visualized by silver staining (Bloom *et al.*, 1987) or, after transfer to nitrocellulose filters, by staining with Coomassie Brilliant Blue (0.1% in 10% glacial acetic acid, 40% methanol), or with HRPO-labeled primary or secondary antibodies and a luminescent substrate (ECL, Amersham).

Protein sequencing

Protein sequencing was performed by Beckmann Auftragsanalytik, Munich, Germany.

Isolation of p32- and p37-specific cDNA clones and nucleotide sequencing

Primers were synthesized based on the published 5′ and 3′ coding sequence of rat prohibitin (Nuell *et al.*, 1991), and prohibitin-specific cDNA was amplified by PCR from cDNA derived from pre-B cell line 33.1.1$^-$. This cDNA was used to isolate prohibitin-specific clones from a mouse myeloma (J558L) cDNA library, generously supplied by Drs Ehrlich, Müller and Rajewsky. Degenerate primers were synthesized based on the obtained p37-specific peptide sequences. p37-specific cDNA was then amplified by PCR from cDNA derived from the pre-B cell line 33.1.1$^-$, and p37-specific clones were isolated from a cDNA library of the mouse pre-B cell 300-19P8 (Leclercq *et al.*, 1989). The nucleotide sequence was obtained using the dideoxy method.

Northern blotting

RNA was isolated from cell lines by the method of Chomczynski and Sacchi (1987). RNA was then separated by electrophoresis in formaldehyde–agarose gels, and blotted onto uncharged nylon membranes (Biodyne A, Pall) by capillary transfer (Sambrook *et al.*, 1989). p32- and p37-specific mRNAs were detected by hybridization with isolated cDNA from p32- and p37-specific cDNA clones that had been labeled with [^{32}P]dATP by the random priming method (Feinberg and Vogelstein, 1984). After each hybridization and exposure, the radioactive probe was stripped off, and the membrane was re-used for the next hybridization. Control hybridizations to monitor loading were performed with a GAPDH-specific probe for cell lines and with a probe specific for S12 ribosomal protein mRNA for tissues (Ayane *et al.*, 1989).

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References

Abney,E.R. and Parkhouse,R.M.E. (1974) *Nature*, **252**, 600–602.
Ales Martinez,J.E., Warner,G.L. and Scott,D.W. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6919–6923.
Alt,F.W., Bothwell,A.L.M., Knapp,M., Siden,E., Mather,E., Koshland,M. and Baltimore,D. (1980) *Cell*, **20**, 293–301.
Alt,F.W., Blackwell,T.K. and Yancopoulos,G.D. (1987) *Science*, **238**, 1079–1087.
Ayane,M., Nielsen,P. and Kohler,G. (1989) *Nucleic Acids Res.*, **17**, 6722.
Bennett,M.K., Garcia Arraras,J.E., Elferink,L.A., Peterson,K., Fleming,A.M., Hazuka,C.D. and Scheller,R.H. (1993) *Cell*, **74**, 863–873.
Bishop,G.A., Ramirez,L.M. and Koretzky,G.A. (1993) *J. Immunol.*, **150**, 2565–2574.
Bloom,H., Beier,H. and Gross,H.S. (1987) *Electrophoresis*, **8**, 93–99.
Brink,R., Goodnow,C.C., Crosbie,J., Adams,E., Eris,J., Mason,D.Y., Hartley,S.B. and Basten,A. (1992) *J. Exp. Med.*, **176**, 991–1005.
Burton,D.R. (1987) In Calabi,F. and Neuberger,M.S. (eds), *Molecular Genetics of Immunoglobulin*. Elsevier, Amsterdam, pp. 1–50.
Cambier,J.C., Vitetta,E.S., Kettman,J.R., Wetzel,G.M. and Uhr,J.W. (1977) *J. Exp. Med.*, **146**, 107–117.
Cambier,J.C., Ligler,F.S., Uhr,J.W., Kettman,J.R. and Vitetta,E.S. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 432–435.
Cambier,J.C., Justement,L.B., Newell,M.K., Chen,Z.Z., Harris,L.K., Sandoval,V.M., Klemsz,M.J. and Ransom,J.T. (1987) *Immunol. Rev.*, **95**, 37–57.
Cambier,J.C., Bedzyk,W., Campbell,K., Chien,N., Friedrich,J., Harwood,A., Jensen,W., Pleiman,C. and Clark,M.R. (1993) *Immunol. Rev.*, **132**, 85–106.
Carsetti,R., Kohler,G. and Lamers,M.C. (1993) *Eur. J. Immunol.*, **23**, 168–178.
Chen,W.J., Goldstein,J.L. and Brown,M.S. (1990) *J. Biol. Chem.*, **265**, 3116–3123.
Cheng,H.-L., Blattner,F.R., Fitzmaurice,L., Mushinski,J.F. and Tucker,P.W. (1982) *Nature*, **296**, 410–415.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156.
Choongkittaworn,N.M., Kim,K.H., Danner,D.B. and Griswold,M.D. (1993) *Biol. Reprod.*, **49**, 300–310.
Clark,M.R., Campbell,K.S., Kazlauskas,A., Johnson,S.A., Hertz,M., Potter,T.A., Pleiman,C. and Cambier,J.C. (1992) *Science*, **258**, 123–126.
Cliby,W., Sarkar,G., Ritland,S.R., Hartmann,L., Podratz,K.C. and Jenkins,R.B. (1993) *Gynecol. Oncol.*, **50**, 34–37.
Cushley,W. and Harnett,M.M. (1993) *Biochem. J.*, **292**, 313–332.
Early,P., Rogers,J., Davies,M., Calame,K., Bond,M., Wall,R. and Hood,L. (1980) *Cell*, **20**, 313–319.
Era,T., Ogawa,M., Nishikawa,S., Okamoto,M., Honjo,T., Akagi,K., Miyazaki,J. and Yamamura,K. (1991) *EMBO J.*, **10**, 337–342.
Evan,G.I., Wyllie,A.H., Gilbert,C.S., Littlewood,T.D., Land,H., Brooks,M., Waters,C.M., Penn,L.Z. and Hancock,D.C. (1992) *Cell*, **69**, 119–128.
Eveleth,D.D.J. and Marsh,J.L. (1986) *Nucleic Acids Res.*, **14**, 6169–6183.
Fasman,G.D. (1989) *Trends Biochem. Sci.*, **14**, 295–299.
Feinberg,A.P. and Vogelstein,B. (1984) *Anal. Biochem.*, **137**, 266.
Finkelman,F.D., Scher,I., Mond,J.J., Kung,J.T. and Metcalf,E.S. (1982) *J. Immunol.*, **129**, 629–637.
Flaswinkel,H. and Reth,M. (1994) *EMBO J.*, **13**, 83–89.
Franklin,D.S. and Jazwinski,S.M. (1993) *J. Cell Biochem.*, Suppl. 17D, 159.
Gaur,A., Yao,X.R. and Scott,D.W. (1993) *J. Immunol.*, **150**, 1663–1669.
Gold,M.R. and DeFranco,A.L. (1994) *Adv. Immunol.*, **55**, 221–295.
Grand,R.J.A. (1989) *Biochem. J.*, **258**, 625–638.
Haggerty,H.G., Wechsler,R.J., Lentz,V.M. and Monroe,J.G. (1993) *J. Immunol.*, **151**, 4681–4693.
Harnett,M.M., Holman,M.J. and Klaus,G.G. (1989) *Eur. J. Immunol.*, **19**, 1933–1939.
Hombach,J., Leclercq,L., Radbruch,A., Rajewsky,K. and Reth,M. (1988) *EMBO J.*, **7**, 3451–3456.
Iglesias,A., Kopf,M., Williams,G.S., Buhler,B. and Kohler,G. (1991) *EMBO J.*, **10**, 2147–2155.
Iglesias,A., Nichogiannopoulou,A., Williams,G.S., Flaswinkel,H. and Kohler,G. (1993) *Eur. J. Immunol.*, **23**, 2622–2630.
Kim,K.-M. *et al.* (1991) *J. Immunol.*, **146**, 819–825.
Kim,K.-M., Ishigami,T., Hata,D., Higaki,Y., Morita,M., Yamaoka,K., Mayumi,M. and Mikawa,H. (1992) *J.Immunol.*, **148**, 29–34.
Kim,K.-M., Alber,G., Weiser,P. and Reth,M. (1993a) *Immunol. Rev.*,

132, 125–146.
Kim,K.-M., Alber,G., Weiser,P. and Reth,M. (1993b) *Eur. J. Immunol.*, **23**, 911–916.
Kim,K.-M., Adachi,T., Nielsen,P.J., Terashima,M., Lamers,M.C., Köhler,G. and Reth,M. (1994) *EMBO J.*, **13**, in press.
Kitamura,D., Roes,J., Kuhn,R. and Rajewsky,K. (1991) *Nature*, **350**, 423–426.
Klaus,G.B., Bijsterbosch,M.K., O'Garra,A., Harnett,M.M. and Rigley,K.P. (1987) *Immunol. Rev.*, **99**, 19–38.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lanzavecchia,A. (1990) *Annu. Rev. Immunol.*, **8**, 773–793.
Law,D.A., Chan,V.W.F., Datta,S.K. and DeFranco,A.L. (1993) *Curr. Biol.*, **3**, 645–657.
Layton,J.E., Pike,B.L., Battye,F.L. and Nossal,G.J.V. (1979a) *J. Immunol.*, **123**, 702–708.
Layton,J.E., Teale,J.M. and Nossal,G.J.V. (1979b) *J. Immunol.*, **123**, 709–713.
Leclercq,L., Butkeraitis,P. and Reth,M. (1989) *Nucleic Acids Res.*, **17**, 6809–6819.
Maki,R., Roeder,W., Traunecker,A., Sidman,C., Wabl,M., Raschke,W. and Tonegawa,S. (1981) *Cell*, **24**, 353–365.
Matsuo,T., Nomura,J., Kuwahara,K., Igarashi,H., Inui,S., Hamaguchi,M., Kimoto,M. and Sakaguchi,N. (1993) *J. Immunol.*, **150**, 3766–3775.
Melcher,U., Vitetta,E.S., McWilliams,M., Lamm,M.E., Phillips-Quagliata,J.M. and Uhr,J.W. (1974) *J. Exp. Med.*, **140**, 1427–1431.
Metcalf,E.S., Mond,J.J. and Finkelman,F.D. (1983) *J. Immunol.*, **131**, 601–605.
Mongini,P.K., Blessinger,C., Posnett,D.N. and Rudich,S.M. (1989) *J. Immunol.*, **143**, 1565–1574.
Morris,D.J., Adler,I.D. and Robinson,T.J. (1993) *Genomics*, **15**, 323–331.
Nitschke,L., Kosco,M.H., Kohler,G. and Lamers,M.C. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1887–1891.
Nuell,M.J. *et al.* (1991) *Mol. Cell. Biol.*, **11**, 1372–1381.
Pure,E. and Vitetta,E.S. (1980a) *J. Immunol.*, **125**, 1240–1242.
Pure,E. and Vitetta,E.S. (1980b) *J. Immunol.*, **125**, 420–427.
Reth,M. (1989) *Nature*, **338**, 383–384.
Reth,M. (1992) *Annu. Rev. Immunol.*, **10**, 97–121.
Reth,M., Petrac,E., Wiese,P., Lobel,L. and Alt,F.W. (1987) *EMBO J.*, **6**, 3299–3305.
Roes,J. and Rajewsky,K. (1993) *J. Exp. Med.*, **177**, 45–55.
Rogers,J., Early,P., Carter,C., Calame,K., Bond,M., Hood,L. and Wall,R. (1980) *Cell*, **20**, 303–312.
Roskams,A.J. *et al.* (1993) *J. Cell. Physiol.*, **157**, 289–295.
Sakaguchi,N., Matsuo,T., Nomura,J., Kuwahara,K., Igarashi,H. and Inui,S. (1993) *Adv. Immunol.*, **54**, 337–392.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanchez,M., Misulovin,Z., Burkhardt,A.L., Mahajan,S., Costa,T., Franke,R., Bolen,J.B. and Nussenzweig,M. (1993) *J. Exp. Med.*, **178**, 1049–1055.
Sato,T. *et al.* (1992) *Cancer Res.*, **52**, 1643–1646.
Scott,D.W. (1978) *Eur. J. Immunol.*, **8**, 13–16.
Smith,S.A., Easton,D.F., Evans,D.G. and Ponder,B.A. (1992) *Nature Genet.*, **2**, 128–131.
Tisch,R., Roifman,C.M. and Hozumi,N. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6914–6918.
Vitetta,E.S. and Uhr,J.W. (1975) *Science*, **189**, 964–969.
Vitetta,E.S., Melcher,U., McWilliams,M., Lamm,M.E., Phillips-Quagliata,J.M. and Uhr,J.W. (1975) *J. Exp. Med.*, **141**, 206–215.
Von Heyne,G. (1985) *J. Mol. Biol.*, **184**, 99–105.
Wenger,R.H., Ayane,M., Bose,R., Kohler,G. and Nielsen,P.J. (1991) *Eur. J. Immunol.*, **21**, 1039–1046.
White,J.J. *et al.* (1991) *Genomics*, **11**, 228–230.
Williams,G.T., Peaker,C.J.G., Patel,K.J. and Neuberger,M.S. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 474–478.
Yuan,D. (1986) *Mol. Cell. Biol.*, **6**, 1015–1022.
Yuan,D. and Tucker,P.W. (1984) *J. Immunol.*, **132**, 1561–1565.
Zan-Bar,I. and Barzilay,M. (1982) *Eur. J. Immunol.*, **12**, 838–844.
Zan-Bar,I., Strober,S. and Vitetta,E.S. (1979) *J. Immunol.*, **123**, 925–930.

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