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OCA-B integrates B cell antigen receptor-, CD40L- and IL 4-mediated signals for the germinal center pathway of B cell development

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Many of the key decisions in lymphocyte differentiation and activation are dependent on integration of antigen receptor and co-receptor signals. Although there is significant understanding of these receptors and their signaling pathways, little is known about the molecular requirements for signal integration at the level of activation of gene expression. Here we show that in primary B cells, expression of the B-cell specific transcription coactivator OCA-B (also known as OBF-1 or Bob-1) is regulated synergistically by the B-cell antigen receptor, CD40L and interleukin signaling pathways. Consistent with the requirement for multiple T cell-dependent signals to induce OCA-B, we find that OCA-B protein is highly expressed in germinal center B cells. Accordingly, germinal center formation is blocked completely in the absence of OCA-B expression in B cells, whereas the helper functions of OCA-B-deficient T cells are indistinguishable from controls. The requirement for OCA-B expression in B cells is germinal center specific since the development of primary B cell follicles, the marginal zone and plasma cells are all intact. Thus, OCA-B is the first example of a transcriptional coactivator that is both synergistically induced by and required for integration of signals that mediate cell fate decisions.

Keywords: coactivator/germinal center/knockout mice/ OCA-B (OBF-1, Bob-1)/signal transduction

Introduction

Immunoglobulin (Ig) gene transcription is specific to B lymphocytes and is regulated by B-cell specific promoter and enhancer elements (Staudt and Lenardo, 1991; Ernst and Smale, 1995). The activity that confers cell-specific promoter function was first identified biochemically as the B-cell specific coactivator OCA-B (Luo *et al*., 1992). OCA-B interacts with the octamer-binding family of transcription factors, Oct 1 and Oct 2, and together with either of these DNA-binding activators mediates efficient cell type-specific transcription of Ig promoters both *in vitro* and in transfected tissue culture cells (Luo *et al*., 1992; Gstaiger *et al*., 1995; Luo and Roeder, 1995; Strubin *et al*., 1995). Biochemical purification and molecular cloning revealed that OCA-B consists of a single proline-rich polypeptide with sequence homology to other coactivators (Luo and Roeder, 1995). Human cDNAs encoding the same protein (designated as OBF-1 or Bob-1) were cloned independently by yeast one-hybrid screens for factors that coactivate Oct 1 and Oct 2 (Gstaiger *et al*., 1995; Strubin *et al*., 1995).

OCA-B-enhanced transcription is dependent upon either Oct 1 or Oct 2 but appears to be restricted to Ig promoters, since OCA-B has no significant effects on transcription of other (e.g. histone H2B) octamer-containing and Oct 1-dependent promoters (Luo *et al*., 1992; Luo and Roeder, 1995). OCA-B alone does not bind specifically to DNA, but instead is recruited to the octamer sites of Ig promoters by direct interaction with the DNA recognition (POU) domain of Oct 1 or Oct 2 (Gstaiger *et al*., 1995; Luo and Roeder 1995; Pfisterer *et al*., 1995; Strubin *et al*., 1995). The binary complex of the octamer-binding activator Oct 1 (or Oct 2) and the coactivator OCA-B is thought to activate transcription initiation by specific interactions with the TATA-box-associated basal transcription machinery (Luo and Roeder, 1995; Schubart *et al*., 1996b). Promoter selectivity is believed to be a complex function of promoter element and basal transcription machinery topology (Luo and Roeder, 1995).

Surveys of a large number of human and murine cell lines indicated that OCA-B mRNA is expressed primarily by cells of B lymphoid origin. High constitutive levels of OCA-B expression were found in transformed cell lines representing all stages of B lymphocyte development, including pro-B, pre-B, mature B and plasma cell stages (Gstaiger *et al*., 1995; Luo and Roeder, 1995; Strubin *et al*., 1995; Schubart *et al*., 1996b). The only known exceptions are Jurkat T cells and immature thymocytes that were found to express OCA-B transiently after stimulation with a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin (Zwilling *et al*., 1997). However, the physiological significance of induced expression in Jurkat cells and thymocytes was not clear (Zwilling *et al*., 1997).

Based on *in vitro* and *in vivo* functional assays and the pattern of expression in cell lines, it was expected that disruption of the *OCA-B* gene would impair both B cell development and Ig transcription. Somewhat surprisingly, however, *OCA-B*$^{-/-}$ mice showed no defect in either early B cell differentiation or IgM production. Instead, the two most prominent features of the mutant phenotype were the absence of germinal centers (GCs) and a profound deficiency in serum Igs of secondary isotypes (Kim *et al*., 1996; Nielsen *et al*., 1996; Schubart *et al*., 1996a). Similar phenotypes have also been reported in *CD40L* (Renshaw *et al*., 1994; Xu *et al*., 1994), *CD40* (Kawabe *et al*., 1994), *CD19* (Engel *et al*., 1995; Rickert *et al*., 1995), *CR2*/*CD21* (Ahearn *et al*., 1996), *interleukin receptor common*

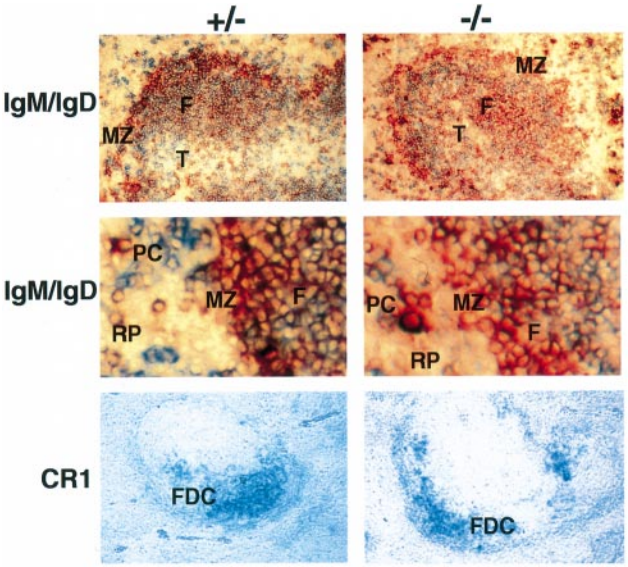

Fig. 1. Spleen lymphoid follicle structure in *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ mice. Spleen sections from matched *OCA-B*$^{+/-}$ (+/–) and *OCA-B*$^{-/-}$ (–/–) littermates were stained with either anti-IgM + anti-IgD (IgM/IgD, red and blue, respectively) or anti-CR1 (mAb 8C12, blue). Magnification: ×40 top, ×160 middle, ×40 bottom. The T cell zone (T), B cell follicle (F), marginal zone (MZ), red pulp area (RP), IgM plasmacytes (PC) and follicular dendritic cell network (FDC) are indicated.

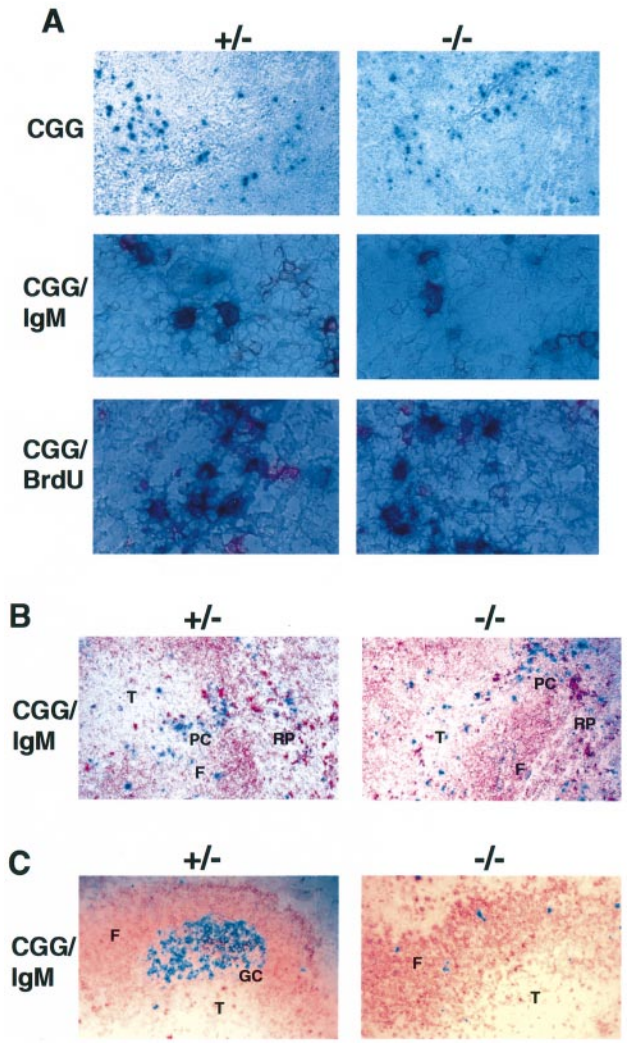

Fig. 2. Kinetic studies of antigen-induced B cell differentiation in *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ mice. (**A**) Spleen sections from *OCA-B*$^{+/-}$ (+/–) and matched *OCA-B*$^{-/-}$ (–/–) littermates that had been immunized with NP-CGG for 3 days were stained with either CGG (blue, ×60), CGG + anti-IgM (CGG/IgM, blue and red, respectively, ×200 from T cell zone) or CGG + anti-BrdU (CGG/BrdU, blue and red, ×200 from T cell zone). (**B**) Spleen sections from the animals that had been immunized with NP-CGG for 5 days were stained with CGG + anti-IgM (CGG/IgM, blue and red, respectively, ×40) to show antigen-specific plasma cells in the T cell area and the red pulp. (**C**) Spleen sections from the animals that had been immunized with NP-CGG for 10 days were stained with CGG + anti-IgM (CGG/IgM, blue and red, respectively, ×40) to reveal antigen-specific germinal centers. The T cell zone (T), B cell follicle (F), red pulp (RP), antigen-specific plasma cells (PC) and germinal center (GC) are indicated.

γ chain (γ_c) (Cao *et al*., 1995; DiSanto *et al*., 1995) and *CD28* (Ferguson *et al*., 1996) mutant mice and humans. However, there is no known link between OCA-B and the B-cell antigen receptor (BCR), interleukin receptor or CD40 pathways, and no understanding of how these signals might be integrated in B cells undergoing the GC reaction.

Here we report that the absence of GCs in *OCA-B*$^{-/-}$ mice may be explained by the finding that signals essential for GC formation, including those produced by the BCR, CD40, and IL 4R, are integrated to induce OCA-B expression specifically in GC cells *in vivo*.

Results

OCA-B is only essential for the GC pathway of B cell differentiation

To determine whether the absence of GCs in *OCA-B*$^{-/-}$ mice was due to alterations in lymphoid microarchitecture, we stained spleen sections with anti-IgM and anti-IgD. We found no reproducible differences either in the primary follicle (IgM^+IgD^+ double positive cells) or in the marginal zone ($IgM^{high}IgD^{low}$ cells) between *OCA-B*$^{-/-}$ and control mice (Figure 1, top and middle panels). Primary B cell follicles in *OCA-B*$^{-/-}$ mice also displayed a normal number and distribution of follicular dendritic cells, as determined with anti-CR1 monoclonal antibody 8C12 (Kinoshita *et al*., 1988) (Figure 1, bottom panel).

There are two pathways of B cell differentiation after immunization with T-dependent antigens (MacLennan, 1994; Nossal, 1994; Kelsoe, 1996). Activated B cells form pauciclonal aggregates which either become plasmacytes or translocate to the follicular area to develop into GC cells (Jacob *et al*., 1991; Liu *et al*., 1991; Lalor *et al*., 1992). To determine which step in T-dependent immune responses requires OCA-B, we immunized *OCA-B*$^{-/-}$ and control mice with nitrophenyl-acetyl chicken gamma globulin (NP-CGG) and followed antigen-specific B cell development *in situ*. We found that the initial proliferative expansion of antigen-specific B cells at day 3 to 6 was similar in *OCA-B*$^{-/-}$ mice and controls (Figure 2A, top panel, only the day 3 data are shown). IgM-positive antigen-specific dividing B cells were found in small aggregates in the T cell zone in all samples (Figure 2A, second panel: IgM^+CGG^+, and third panel: CGG^+BrdU^+). At day 5 to day 10, similar frequencies of antigen-specific plasmacyte foci were detected at the outer

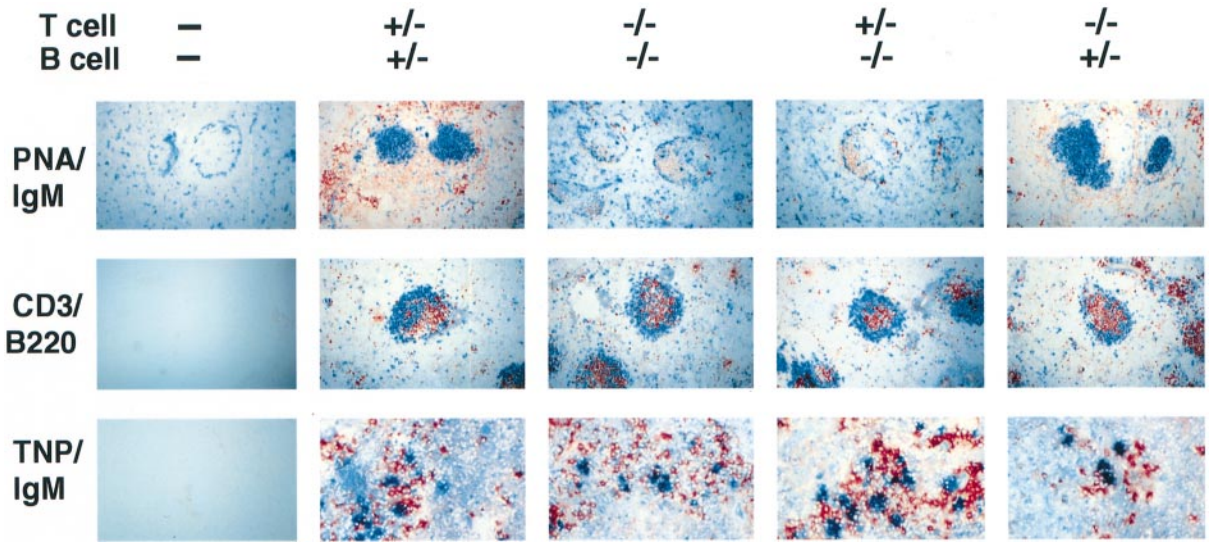

Fig. 3. Germinal center and plasma cell development in Rag-1-deficient mice reconstituted with *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ B and T cells. Spleen cryosections were prepared from the reconstituted mice [the combinations of *OCA-B*$^{+/-}$ (+/–) and *OCA-B*$^{-/-}$ (–/–) B and T cells are indicated on the top] as well as the unreconstituted Rag-1$^{-/-}$ control (–) that had been immunized with TNP-KLH for 14 days. The sections were double stained with either PNA + anti-IgM (top panel, PNA/IgM, blue and brown, respectively, ×40) for GC and follicular B cells, CD3 + anti-B220 (middle panel, CD3/B220, brown and blue, respectively, ×40) for T and B cells in lymphoid follicles or TNP-AP + anti-IgM (bottom panel, TNP/IgM, blue and brown, respectively, ×80) for antigen-specific and IgM positive plasma cells in the red pulp.

edge of the T cell zone adjacent to the red pulp area in both *OCA-B*$^{-/-}$ and control mice (Figure 2B, day 5). However, follicular expansion of the antigen-activated B cells and formation of nascent GCs were completely absent in the mutant mice (Figure 2C, day 10). Similar results were also obtained with spleens from mice immunized with trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) that were stained to reveal hapten-reactive B cells (not shown). We conclude that the absence of OCA-B does not affect either the initial response to antigen, primary B-cell follicle formation, marginal zone structure or plasma cell differentiation, but does block specifically GC development inside the follicle.

B-cell autonomous defect in GC development

To determine whether the absence of GCs and secondary isotype antibody responses in *OCA-B*$^{-/-}$ mice is T- or B-cell specific, we performed short-term reconstitution experiments using lymphocyte-deficient *RAG-1*$^{-/-}$ mice as recipients of wild-type, *OCA-B*$^{+/-}$ or *OCA-B*$^{-/-}$ B and T cells. Purified B and T cells were combined and injected into *RAG-1*$^{-/-}$ mice, and a day after cell transfer the reconstituted animals were challenged with TNP-KLH. Spleens from the reconstituted animals were analyzed by immunohistochemistry, and sera were collected to measure specific antibody responses 14 days after antigen challenge. Results for wild-type and *OCA-B*$^{+/-}$ T and B cells were identical, and only experiments with *OCA-B*$^{+/-}$ cells are shown.

All mice reconstituted with T and B cells developed specific anti-TNP IgM responses (not shown), and showed similar numbers of anti-TNP-specific plasma cells in the red pulp of the spleen (Figure 3, bottom panel, TNP-alkaline phosphatase and anti-IgM staining). In addition, all combinations of B and T cells produced normal appearing lymphoid follicles with distinct T and B cell areas (Figure 3, middle panel, anti-CD3 and anti-B220 staining). However, only those mice receiving wild-type

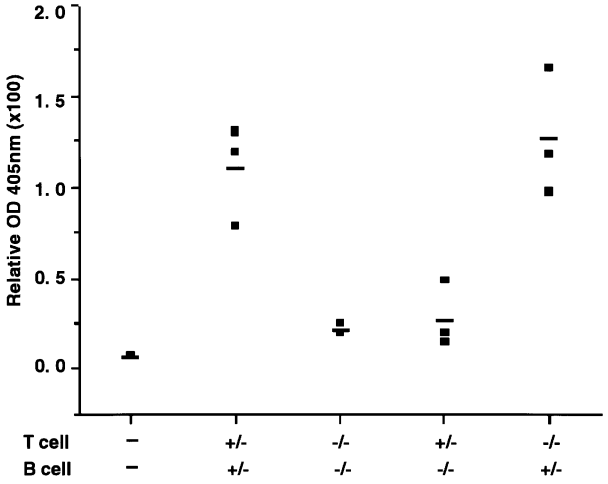

Fig. 4. Anti-TNP-specific IgG antibody responses in Rag-1-deficient mice reconstituted with *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ B and T cells. Sera were collected from the reconstituted mice [the combinations of *OCA-B*$^{+/-}$ (+/–) and *OCA-B*$^{-/-}$ (–/–) B and T cells are indicated at the bottom] as well as the unreconstituted Rag-1$^{-/-}$ control (–) that had been immunized with TNP-KLH for 14 days. Anti-TNP IgG titers were determined by TNP-specific ELISA. Results from individual animals are represented by the solid squares, and the mean values are indicated by horizontal bars.

B cells produced GCs and anti-TNP-specific IgG responses (Figure 3, top panel, PNA and anti-IgM staining, and Figure 4). Mice reconstituted with *OCA-B*$^{-/-}$ B cells and either *OCA-B*$^{+/-}$ or *OCA-B*$^{-/-}$ T cells did not develop GCs or anti-TNP IgG antibodies (Figure 3, top panel, PNA and anti-IgM staining, and Figure 4). In contrast, *OCA-B*$^{+/-}$ or *OCA-B*$^{-/-}$ T cells were equally effective in producing the helper functions for IgG anti-TNP responses and GC formation. Thus, the absence of GCs and IgG antibody responses in *OCA-B*$^{-/-}$ mice is a B-cell autonomous defect, and *OCA-B*$^{-/-}$ T cells are indistinguishable from wild-type T cells in supporting GC formation and antibody responses *in vivo*.

OCA-B is expressed preferentially in GC B cells

The restricted nature of the *OCA-B*$^{-/-}$ phenotype was difficult to account for in view of the reported lack of regulated *OCA-B* expression in cell lines representing all stages of B cell differentiation (Gstaiger *et al*., 1995; Luo and Roeder, 1995; Strubin *et al*., 1995; Schubart *et al*., 1996b). To determine where *OCA-B* is expressed *in vivo*, we performed immunohistochemical staining of peripheral lymphoid organs from naive and immunized mice with two different affinity-purified polyclonal rabbit antibodies to OCA-B (Figure 5A). In all experiments, *OCA-B*$^{+/+}$ and *OCA-B*$^{+/-}$ mice were indistinguishable, and only the results for *OCA-B*$^{+/-}$ are shown. In contrast to the broad distribution of OCA-B in transformed B cell lines (Gstaiger *et al*., 1995; Luo and Roeder, 1995; Strubin *et al*., 1995; Schubart *et al*., 1996b), *OCA-B* expression in peripheral lymphoid tissues was restricted to a subset of B cells that were in the IgM-positive and CD3-negative compartments of the white pulp (Figure 5A, bottom panels) and co-localized with GC-specific peanut agglutinin (PNA) staining (Figure 5A, top and middle panels of the serial sections). OCA-B-expressing cells were seen throughout the GCs but were most prominent in what appeared to be GC dark zones that contain rapidly proliferating centroblasts (Figure 5A). We were unable to detect OCA-B staining in activated B cell blasts in the T cell zone or in plasmacytes in the red pulp. Neither OCA-B nor PNA staining was seen in the *OCA-B*$^{-/-}$ controls (Figure 5A).

To confirm the results of the immunohistology, spleen B cells from immunized mice were separated into high and low density populations, and OCA-B protein expression was measured by Western blotting. GC B cells differ from naive B cells in that they are activated and rapidly proliferating (MacLennan, 1994; Nossal, 1994; Kelsoe, 1996). These cells are enriched in the low density B cell fractions after Percoll gradient centrifugation, whereas naive B cells are found in high density fractions. Anti-OCA-B antibodies (Luo and Roeder, 1995) recognize two protein bands of 34 and 35 kDa in both human and mouse B cells (Luo and Roeder, 1995; X.-F.Qin, unpublished). The 35 kDa species is a post-translationally modified form of the 34 kDa protein, and the two isoforms are equally active in stimulating octamer-dependent transcription from Ig promoters (Luo and Roeder, 1995). Consistent with the immunolocalization experiments, OCA-B was highly expressed in the low density activated B cell fraction but difficult to detect in high density resting B cells or T cell controls (Figure 5B). Similarly, OCA-B protein was abundant in FACS-sorted IgD^{-}CD38^{+} human tonsil GC B cells (Liu *et al*., 1995) but present at only low levels in IgD^{+}CD38^{-} naive B cells (not shown).

Like GC B cells, pre-B cells which have acquired a functional Ig H chain are rapidly dividing cells (Rolink and Melchers, 1993; Melchers *et al*., 1994). To determine whether *OCA-B* is also expressed during early B cell development, we measured OCA-B protein expression in large dividing B220^{+}IgM^{-} pro-/pre-B cells and small resting B220^{+}IgM^{+} maturing B populations (Melchers *et al*., 1994; Constantinescu and Schlissel, 1997). Low levels of both 34 and 35 kDa OCA-B bands were detected in the purified bone marrow B cells (Figure 6, lanes 1–4). The level of OCA-B protein was 2- to 3-fold higher in the B220^{+}IgM^{-} pro-/pre-B populations than in the B220^{+}IgM^{+} maturing B as measured by densitometry. When compared with activated spleen B cells (Figure 6, lanes 6 and 7), the amounts of OCA-B present in the pro-/pre-B cells was 10–15 times lower on a per cell basis. We conclude that low levels of OCA-B are expressed in bone marrow B cell precursors, although OCA-B is not essential for early B cell differentiation since B cell development and maturation are normal in *OCA-B*$^{-/-}$ mice (Kim *et al*., 1996; Nielsen *et al*., 1996; Schubart *et al*., 1996a).

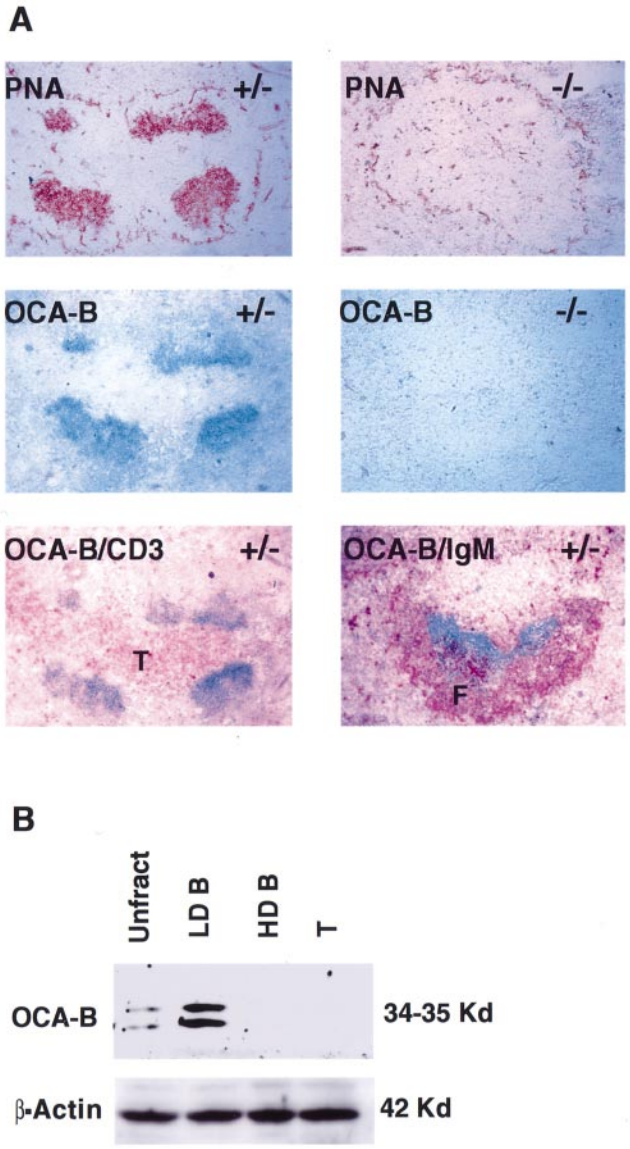

Fig. 5. (**A**) Preferential expression of *OCA-B* in GC B cells. Spleen sections from *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ mice that had been immunized with NP-CGG for 10 days were stained with either PNA (PNA, brown), affinity-purified rabbit anti-OCA-B (OCA-B, blue), anti-OCA-B + anti-CD3 (OCA-B/CD3, blue and red, respectively) or anti-OCA-B + anti-IgM (OCA-B/IgM, blue and red, respectively). The T cell zone (T) and B cell follicle (F) are indicated. Magnification ×40. (**B**) Low density B cells contain high levels of OCA-B protein. Whole-cell extracts (15 μg proteins) prepared from Percoll gradient-fractionated low density B cells (LD B) and high density B cells (HD B) were analyzed for OCA-B expression by Western blotting. Unfractionated splenocytes (Unfract) and purified T cells (T) were included as controls. OCA-B protein bands (34–35 kDa doublets) are indicated. β-Actin Western blotting was performed to ensure that an equivalent amount of proteins was present in each of the samples.

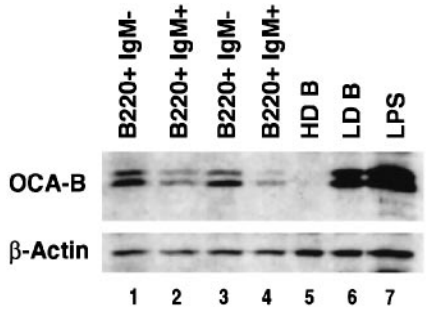

Fig. 6. OCA-B expression in early and late B cells. Bone marrow B cells from young C57/BL6 mice were sorted into large $B220^{+}IgM^{-}$ (B220+IgM–) and small $B220^{+}IgM^{+}$ (B220+IgM+) populations (see Materials and methods). Samples prepared from two independent sortings were analyzed. High density (HD B), low density (LD B) and LPS-stimulated (day 3) splenic B cell samples were as described in Figures 5 and 7. Western blotting was performed on SDS whole-cell lysates (see Materials and methods). Lysates made from 2×10^5 cells were used for the bone marrow and HD B samples; whereas 5×10^4 cells were used for LD B and LPS samples. β-Actin Western blotting was included as a control.

Taken together, B cells that express high levels of OCA-B are restricted to GCs, and this restricted pattern of OCA-B expression correlates with its functional requirement in a cell-autonomous manner for GC development *in vivo*.

Induction of OCA-B in mature B cells

Genetic experiments have shown that GC formation requires integration of BCR, CD40 and cytokine receptor signaling pathways (Kawabe *et al*., 1994; Xu *et al*., 1994; Cao *et al*., 1995; Rickert *et al*., 1995; Ahearn *et al*., 1996; Ferguson *et al*., 1996). To determine whether *OCA-B* expression is regulated by these signals, we measured *OCA-B* expression in primary B cells stimulated with various activators. High levels of OCA-B mRNA and protein, comparable with those detected in many B cell lines, were induced in resting B cells by lipopolysaccharide (LPS), a polyclonal activator. Induction of mRNA and protein was first seen 12 h after stimulation, and progressively accumulated over a 7 day culture period (Figure 7A and B). Although the protein and mRNA induction follow a similar time course, the level of OCA-B protein induction is higher than that of its mRNA (Figure 7A and B). Thus both transcriptional and translational processes are likely to be involved in *OCA-B* regulation. Consistent with the finding that the 35 kDa form of the OCA-B protein is derived from the 34 kDa form through post-translational modification (Luo and Roeder, 1995), the induction and accumulation of the modified species appear to be slower than those of its precursor.

In contrast to LPS, individual physiological stimuli such as CD40L, BCR engagement (anti-μ cross-linking) and IL 4 induced only low levels of *OCA-B* expression (Figure 7C). During T-cell dependent immune responses *in vivo*, these stimuli are integrated to activate B cell differentiation. To determine whether OCA-B levels could be regulated coordinately by these physiological stimuli, we measured *OCA-B* expression in response to combinations of CD40L, anti-μ and IL 4 stimulation. High levels of *OCA-B* expression were seen with either CD40L + IL 4, CD40L + anti-μ or CD40L + anti-μ + IL 4 (Figure 7C, lanes 8–10), but not with anti-μ + IL 4

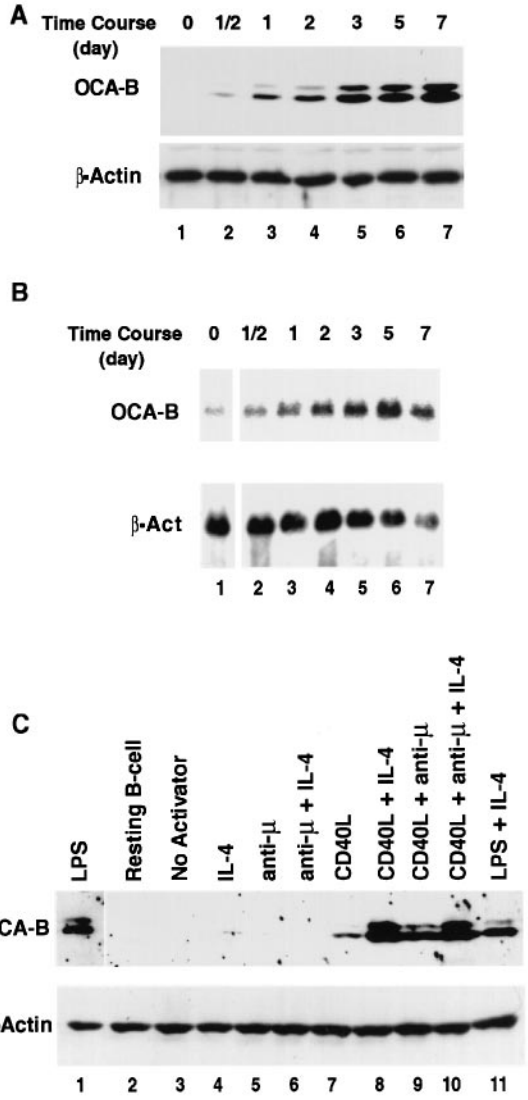

Fig. 7. (**A**) Western blot analysis of OCA-B protein expression in primary B cells stimulated with LPS. Percoll gradient-purified high density B cells were cultured with 25 μg/ml LPS for 1/2, 1, 2, 3, 5 and 7 days as indicated. Samples from unstimulated high density B cells were included as time zero (0) controls. A 7 μg aliquot of whole-cell extracts was used in the experiment. β-Actin Western blotting was included as a control. (**B**) Northern blot analysis of OCA-B mRNA expression in primary B cells stimulated with LPS. β-Actin (β-Act) was used as a control for mRNA loading. OCA-B protein and mRNA signals are indicated. (**C**) Synergistic induction of OCA-B by anti-μ, CD40L and IL 4. Percoll gradient-purified high density B cells were cultured with stimulators as indicated for 3 days. OCA-B protein expression was analyzed by Western blotting (7 μg of whole-cell extracts). Day 3 LPS and LPS + IL 4 samples were included for comparison. β-Actin Western blotting was included as a control.

(Figure 7C, lane 6), with a time course similar to that seen for LPS stimulation (Figure 7C; data not shown). The kinetics of OCA-B induction are consistent with the specificity of the anti-OCA-B antibody staining in lymphoid tissues. OCA-B expression was below the level of immunohistochemical detection in recently activated B cells that are found in the T area (Figure 5A). In contrast, high levels of anti-OCA-B staining were seen in GC B cells that develop several days after stimulation with antigen and T cell help (Figure 5A). We conclude

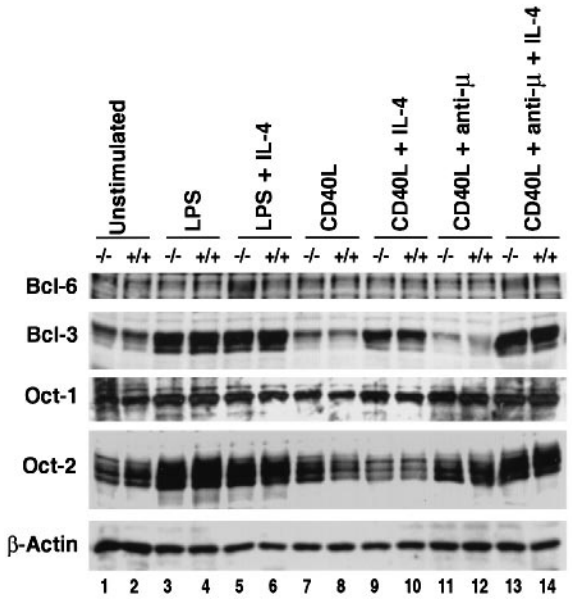

Fig. 8. No alteration of Bcl-6, Bcl-3, Oct 1 and Oct 2 protein expression in OCA-B mutant B cells. T cell-depleted splenic B cells (see Materials and methods) from OCA-B$^{-/-}$ (–/–) mice and the wild-type control (+/+) were cultured with stimulators as indicated for 3 days. Whole-cell extracts (10 μg proteins) were used for Western blot analysis. The blots were probed with antibodies against Bcl-6, Bcl-3, Oct 1, Oct 2 and β-actin. The protein bands with the expected molecular weight are indicated.

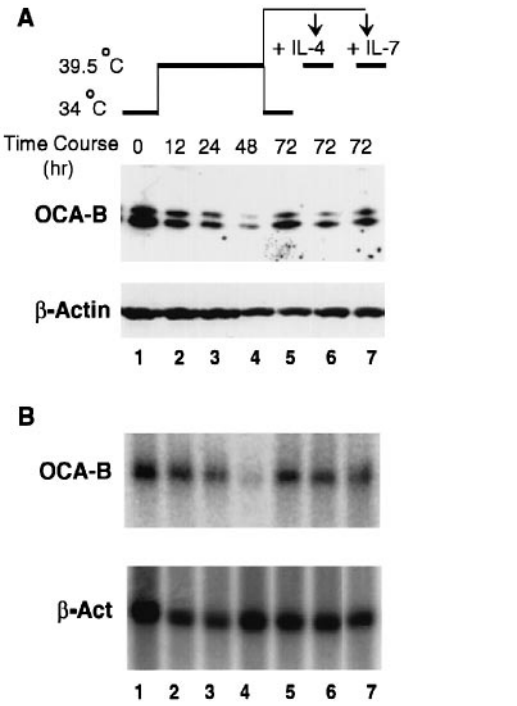

Fig. 9. v-Abl tyrosine kinase activates OCA-B expression. ts-Abl pre-B cells (103/4) were grown at the permissive temperature (34°C, lane 1), and shifted to the non-permissive temperature (39.5°C) for 12, 24 and 48 h (lanes 2, 3 and 4), then either shifted back to 34°C for 24 h (lane 5), or kept at the non-permissive temperature for another 24 h with addition of 5 ng/ml IL 4 (lane 6) or 15 ng/ml IL-7 (lane 7). (**A**) Western blot analysis of OCA-B protein levels (10 μg of whole-cell extracts). β-Actin was also probed as a control. (**B**) Northern blot analysis of OCA-B mRNA expression. β-Actin (β-Act) was used as a control.

that *OCA-B* expression can be modulated by a number of signaling pathways, each of which is essential for GC formation *in vivo*.

OCA-B knockout does not alter the expression of Bcl-3, Bcl-6 and Oct 2

Recent studies have shown that GC formation is also disrupted in *Bcl-3* and *Bcl-6* knockout mice (Dent *et al*., 1997; Franzoso *et al*., 1997; Fukuda *et al*., 1997; Schwarz *et al*., 1997). In particular, *Bcl-6* is found to be expressed preferentially in GC B cells and is required in a cell-autonomous manner for GC development (Fukuda *et al*., 1997). To determine whether the *OCA-B* mutation affects either *Bcl-3* or *Bcl-6* expression, we performed Western blotting experiments to measure Bcl-3 and Bcl-6 protein levels in *OCA-B*$^{-/-}$ splenic B cells that were stimulated with combinations of B cell activators. In the absence of OCA-B, neither baseline expression nor induction of Bcl-3 and Bcl-6 proteins was altered. A low level of Bcl-6 protein was detected in all the samples (Figure 8); no apparent induction was observed with the treated cells (Figure 8, lanes 3–14), which is consistent with the previous reports that Bcl-6 is not induced by LPS, anti-IgM, CD40L, IL 4 or combinations of these signals (Allman *et al*., 1996; Fukuda *et al*., 1997). In contrast, Bcl-3 was induced modestly by LPS, LPS + IL 4, CD40L + IL 4 and CD40L + anti-μ + IL 4 (Figure 8, lanes 3–6, 9, 10, 13 and 14), but no induction was found with CD40L alone or CD40L + anti-μ treatments (Figure 8, lanes 7, 8, 11 and 12). In all conditions, the levels of Bcl-3 expression were equivalent in *OCA-B*$^{-/-}$ B cells and the wild-type controls. To examine whether the OCA-B mutation alters Oct 1 and Oct 2 protein levels, the Western blots were probed with anti-Oct 1 and anti-Oct 2 antibodies. Oct 1 protein was found to be slightly increased in all the stimulated B cell samples (Figure 8, lanes 3–14), whereas Oct 2 was induced modestly by LPS, LPS + IL 4, CD40L + anti-μ and CD40L + anti-μ + IL 4 treatments (Figure 8, lanes 3–6 and 11–14), but not CD40L or CD40L + IL 4 treatments (Figure 8, lanes 7–10). OCA-B$^{-/-}$ B cells were identical to the wild-type in terms of Oct 1 and Oct 2 expression under all conditions tested. Taken together, we conclude that *OCA-B* disruption does not affect the expression of either *Bcl-3*, *Bcl-6* or *Oct 2* which have been implicated in late B cell differentiation and GC development.

Regulation of OCA-B expression by v-Abl kinase in pre-B cells

The regulated expression of *OCA-B* in primary B cells contrasts with the observation that transformed cell lines representing all stages of B cell development contain high levels of OCA-B protein. To determine whether oncogenic transformation *per se* can activate *OCA-B* expression, we made use of a temperature-sensitive Abelson virus (ts-Abl)-transformed pre-B cell line (Chen *et al*., 1994). High levels of OCA-B were found in the 103/4 ts-Abl cells at the permissive temperature when v-Abl was active (Figure 9A and B, lane 1). However, shifting the temperature to non-permissive conditions resulted in downregulation of OCA-B at both protein and RNA levels (Figure 9A and B, lanes 2, 3 and 4). Further, when the cells were returned to the permissive temperature, OCA-B expression

was largely restored (Figure 9A and B, lane 5). The v-Abl protein binds to and constitutively activates Jak-1 and Jak-3 kinases (Danial *et al*., 1995). These kinases normally are activated by interleukin receptor common γ_c cross-linking, and they in turn activate Stat-5 and Stat-6 (Darnell *et al*., 1994; Ihle, 1996). To determine whether *OCA-B* is downstream of the γ_c family of interleukin receptors in pre-B cells, we stimulated the 103/4 ts-Abl cells at the non-permissive temperature with either IL 4 or IL-7. Both IL-7 and IL 4 could partially restore *OCA-B* expression in the ts-Abl-transformed cells (Figure 9A and B, lanes 6 and 7). Thus, both of these cytokines can induce OCA-B through the interleukin receptor common γ_c and the Jak–STAT pathway.

Discussion

Taken together, our results reveal that expression of *OCA-B* is induced synergistically by signals emanating from the BCR, CD40 and IL 4 receptor. This results in a pattern of expression which is both GC B cell specific and consonant with a B cell-autonomous GC deficiency in *OCA-B*$^{-/-}$ mice.

T cell-dependent antibody responses are initiated in the T cell zone of the white pulp with a proliferative burst followed by B cell differentiation into either GC cells or short-lived plasma cells (MacLennan, 1994; Nossal, 1994; Kelsoe, 1996). The GC pathway produces long-lived memory B cells that can produce high-affinity antibodies of secondary isotypes. In contrast, B cells that differentiate directly into plasmacytes tend to produce low-affinity primary IgM antibodies (MacLennan, 1994; Nossal, 1994; Kelsoe, 1996). We and others have reported that inactivation of *OCA-B* in mice results in disruption of antigen-dependent immune responses and GC formation (MacLennan, 1994; Nossal, 1994; Kelsoe, 1996). However, it was not clear from those studies whether the defects are B cell autonomous or whether other aspects of T-dependent B cell responses are also disrupted by the *OCA-B* mutation. In particular, the proliferative response to antigen, plasma cell differentiation, primary follicle formation and the establishment of marginal zone B cells were not examined (Kim *et al*., 1996; Nielsen *et al*., 1996; Schubart *et al*., 1996a). The immunohistochemical experiments presented here suggest that *OCA-B* is essential only for the GC reaction, whereas other B cell responses are *OCA-B* independent. Thus, although GC and extrafollicular B cell differentiation pathways are both induced in response to antigen, these responses appear to be governed by different sets of transcription regulators. This notion is also supported by experiments with *Bcl-6*$^{-/-}$ mice (Fukuda *et al*., 1997). Bcl-6 differs from OCA-B in that it is a typical DNA-binding transcription factor that belongs to the Krüppel-type zinc finger family whereas OCA-B is a coactivator that does not bind to DNA directly. Nevertheless, like *OCA-B*$^{-/-}$ mice, *Bcl-6*$^{-/-}$ mice have specific abnormalities in GC development (Dent *et al*., 1997; Fukuda *et al*., 1997) and the Bcl-6 protein is also expressed preferentially in GC B cells (Fukuda *et al*., 1997). One of the important differences between the two mutations is that Bcl-6 is not induced by CD40L, anti-IgM or interleukins (Figure 8) (Allman *et al*., 1996; Fukuda *et al*., 1997), and the connection between Bcl-6 and the activation of the GC reaction remains obscure (Fukuda *et al*., 1997). In contrast, OCA-B appears to be directly downstream of the B-cell signaling pathways that activate GC cell differentiation. Since *Bcl-6* expression is not altered in *OCA-B*$^{-/-}$ B cells, it suggests that OCA-B and Bcl-6 are involved in different pathways.

How *OCA-B* is induced by CD40L, anti-IgM and interleukins remains unclear, but the observation that many signaling pathways converge on *OCA-B* is consistent with previous reports that all transformed B cells express this coactivator. The early cytoplasmic mediators activated by CD40L, anti-IgM and interleukins in B cells have been studied extensively, and the three pathways triggered by these receptors are for the most part distinct. Cross-linking the BCR activates src and syk family kinases as well as the ras pathway (Gold and DeFranco, 1994). In contrast, the tumor necrosis factor receptor-associated factors (TRAFs) appear to be the early mediators of the CD40 signaling (Cheng *et al*., 1995; Rothe *et al*., 1995; Ishida *et al*., 1996), while the Jaks and Stats are the primary transducers of IL 4 receptor signals (Darnell *et al*., 1994; Ihle, 1996). Malignant transformation frequently involves deregulated expression or mutation in components of these same pathways. For example, *OCA-B* normally is induced through the Jak–STAT pathway by interleukins and, in Abl-transformed pre-B cells, the oncogenic v-Abl kinase constitutively activates Jak-1 and Jak-3 (Danial *et al*., 1995) leading to *OCA-B* induction. We propose that the disparity between the pattern of OCA-B expression *in vivo* and in tumor cell lines is the result of *OCA-B* induction by the oncogenes that immortalize B cells.

The problem of how the signals emanating from CD40, BCR and interleukin receptors are integrated to produce the GC reaction has not been resolved. OCA-B mRNA and protein expression peaks 72–96 h after B cell stimulation. Thus, *OCA-B* induction is a late response which is likely to be downstream of immediate early B cell activators like NF-κB, NF-AT and AP-1 (Gold and DeFranco, 1994). The nuclear programs induced in activated B cells by these early mediators are diverse, and most B cell responses are not affected by the absence of *OCA-B*. For example, the proliferative responses to LPS and CD40 cross-linking, as well as isotype switching induced by IL 4 *in vitro*, are all normal in *OCA-B*$^{-/-}$ B cells (Kim *et al*., 1996). Although it is still possible that these pathways are integrated by a single common cytoplasmic activator for the GC program, it seems more likely that integration of these signals is a nuclear event. Of the mediators activated by the CD40L, anti-IgM and interleukin pathways, the only one that has been associated with the GC reaction is the p52 subunit of the NF-κB family of transcription factors (Baeuerle and Baltimore, 1996; Baldwin, 1996). p52 has been implicated in the GC reaction through its association with Bcl-3 (Bours *et al*., 1993). *Bcl-3*$^{-/-}$ mice resemble *OCA-B*$^{-/-}$ mice in that they have no GCs (Franzoso *et al*., 1997; Schwarz *et al*., 1997), but the absence of GCs in *Bcl-3*$^{-/-}$ mice differs from the *OCA-B*$^{-/-}$ phenotype in that it is not B cell autonomous (Franzoso *et al*., 1997). In addition, neither baseline expression nor induction of *Bcl-3* in B cells is affected by *OCA-B* disruption (Figure 8). Thus, the GC defect in Bcl-3$^{-/-}$ mice appears to be more closely related to that found in mice that are deficient in tumor necrosis factor-

α (TNF-α), TNF receptor I, lymphotoxin α (LTα) or LTβ (De Togni *et al*., 1994; Le Hir *et al*., 1996; Matsumoto *et al*., 1996; Pasparakis *et al*., 1996). In contrast to the *OCA-B*$^{-/-}$ mice, all of these mice display disrupted lymphoid microarchitecture and abnormal follicular dendritic cell networks (De Togni *et al*., 1994; Le Hir *et al*., 1996; Matsumoto *et al*., 1996; Pasparakis *et al*., 1996). Furthermore, in adoptive transfer experiments, *LT*α- or *Bcl-3*-deficient B cells are able to produce GCs, and thus the GC deficiency in these mice is not B-cell autonomous (Matsumoto *et al*., 1996; Franzoso *et al*., 1997).

Biochemical studies indicate that OCA-B enhances transcription through specific protein–protein interactions with Oct 1 or Oct 2. Oct 2 is expressed predominantly in B cells and, like OCA-B, is induced by several different B cell activators (Figure 8) (Corcoran and Karvelas, 1994). Furthermore, the *Oct 2* mutation also affects late B cell activation and terminal differentiation (Corcoran *et al*., 1993; Corcoran and Karvelas, 1994), but the phenotypes of the *OCA-B*$^{-/-}$ and *Oct 2*$^{-/-}$ mice are not identical. *Oct 2*$^{-/-}$ mice show early post-natal lethality (Corcoran *et al*., 1993) and profound defects in T-cell-independent B cell activation (Corcoran and Karvelas, 1994). For example, *Oct 2*$^{-/-}$ B cells respond poorly to LPS and fail to differentiate into antibody-forming cells (Corcoran and Karvelas, 1994). In contrast, *OCA-B*$^{-/-}$ B cells respond to LPS stimulation and produce normal appearing plasma cell foci (Kim *et al*., 1996). Consistent with the finding that Oct 2 is involved in a distinct set of B cell processes (Corcoran *et al*., 1993; Feldhaus *et al*., 1993; Corcoran and Karvelas, 1994), the pattern of Oct 2 induction by LPS, anti-μ, CD40L, IL 4 and their combinations differs from that of OCA-B. Furthermore, the basal and induced levels of Oct 2 expression are not affected by the disruption of the *OCA-B* gene (Figure 8). The more severe and pleiotropic phenotype of the *Oct 2* knockout is consistent with the hierarchy of activator and coactivator in transcriptional regulation (Luo and Roeder, 1995).

OCA-B is the prototype cell type-specific transcription coactivator, and thus belongs to a class of regulators that generally are thought to be constitutively expressed within the lineage of a given cell type (Gstaiger *et al*., 1995; Luo and Roeder, 1995; Pfisterer *et al*., 1995; Strubin *et al*., 1995; Schubart *et al*., 1996b). The observation that OCA-B is inducible and found predominantly in GC B cells *in vivo* was unexpected. The only other indication that OCA-B might differ from other well-described coactivators in being inducible was the observation that some T cell lines and immature thymocytes can be activated to express OCA-B after stimulation with PMA and ionomycin (Zwilling *et al*., 1997). However, since primary T cells do not appear to express OCA-B and since T cell development and helper T-cell function for antibody responses are completely normal in *OCA-B* mutant mice, the physiological significance of OCA-B induction with pharmacological agents is not clear. In conclusion, activation of *OCA-B* is a late nuclear response that reflects multiple signaling inputs. Irrespective of the site of GC signal integration, it is clear that *OCA-B* is induced synergistically by BCR, CD40 and IL 4R signals and that these pathways converge on *OCA-B* in B cells in a manner which is essential for the GC program. Therefore, coactivator OCA-B resembles conventional DNA-binding transcription factors in integrating signals that mediate cell fate decisions.

Materials and methods

Mice, antigens and immunizations

Matched littermates from *OCA-B*$^{+/-}$ back crosses (F3 in C57/BL6 background) were used in all experiments. No difference was observed between wild-type and heterozygous mutant animals, and only the latter are shown. Mice were maintained in a specific pathogen-free facility. NP-CGG (15:1) was prepared by conjugating (4-hydroxy-3 nitrophenyl)acetyl succinimide ester (Genosys) to CGG (Sigma) as previously described (Lalor *et al*., 1992), and TNP-KLH was prepared by conjugating 2,4,6-trinitrobenezene sulfonic acid (Sigma) to keyhole limpet hemocyanin (Sigma) as previously described (Liu *et al*., 1991). Mice (three sets for each time point) were immunized at the age of 8–12 weeks with 100 mg of alum precipitate of NP-CGG or TNP-KLH given intraperitoneally.

Reconstitution of splenic B and T cells in Rag-1-deficient mice

B and T cell reconstitution in Rag-1-deficient mice was performed as described (Fukuda *et al*., 1997). B cells were purified from splenocytes of *OCA-B*$^{+/-}$ and *OCA-B*$^{-/-}$ matched littermates by depleting non-B lineage cells with biotinylated Mac-1 monoclonal antibody (M1/70, Pharmingen) in conjunction with streptavidin-coated Dynal magnetic beads (SA-M280, Dynal), and with anti-mouse Thy1.2-coated Dynal magnetic beads [Mouse pan-T (Thy1.2), Dynal]. T cells from the same mice were purified using anti-mouse B220-coated Dynal magnetic beads [Mouse pan-B (B220), Dynal] instead of anti-mouse Thy1.2-coated beads. FACS analysis with B220 and IgM, or CD3 staining showed that the B cell preparations contained >93% B220$^+$IgM$^+$ cells and <1% contaminating CD3$^+$ T cells, while T cell preparations contained >95% CD3$^+$ cells and <1% contaminating B220$^+$IgM$^+$ B cells. The mixtures of the purified B and T cells (1.5×10^7 each) from OCA-B$^{+/-}$ and OCA-B$^{-/-}$ animals in four different combinations were injected intravenously into Rag-1-deficient mice. Three independent reconstitutions were performed for each of the B and T cell combinations. The reconstituted mice were challenged with 100 mg of alum precipitate of TNP-KLH given intraperitoneally 24 h after the transfer. The spleen and serum were harvested on day 14 post-immunization.

Immunohistochemistry

Serial spleen cryosections (5–6 μm) were prepared for immunohistochemical staining. Biotinylated PNA (Vector) was used to stain GC B cells and was revealed with either an ABC/alkaline phosphatase and AP/III kit (blue, Vector) or an ABC/peroxidase and DAB kit (brown, Vector) as indicated. Anti-IgM was a direct peroxidase-labeled goat anti-mouse IgG antibody (Sigma) and was developed with AEC (red, Vector) or DAB substrate (brown, Vector). Biotinylated anti-mouse CD3 (145-2C11, Pharmingen) was revealed with ABC/peroxidase and AEC or DAB substrate after TSA amplification (DuPont NEN). Biotinylated anti-mouse B220 (RA3-6B2, Pharmingen) was revealed with an ABC/alkaline phosphatase and AP/III substrate kit (blue, Vector). TNP-conjugated alkaline phosphatase was used to detect the hapten-specific B cells and was visualized with an AP/III substrate kit (blue, Vector), and biotin-labeled CGG was used as the probe to detect the antigen-specific B cells. The number of B cells reacting to the carrier CGG was consistent between the individuals of either heterozygous or homozygous knockout animals. For IgD staining, rat anti-mouse IgD (SBA-1, Southern Biotech) was used and detected with affinity-purified biotinylated rabbit anti-rat IgG antibody (Vector) in conjunction with an ABC/alkaline phosphatase and AP/III substrate kit (blue, Vector). Anti-CR1 (8C12, Pharmingen) was a biotinylated antibody and was visualized with an ABC/alkaline phosphatase and AP/III substrate kit (blue, Vector). *In vivo* bromodeoxyuridine (BrdU) pulse-labeling and detection were performed as described (Toellner *et al*., 1996). Mouse anti-BrdU (BU20a, Dako) was used as the first reagent and revealed with biotinylated rat anti-mouse IgG1 (G1-6.5, Pharmingen) and ABC/alkaline phosphatase.

Anti-OCA-B antibodies were affinity purified from rabbit antisera raised against the 172–256 region of OCA-B (Luo and Roeder 1995) or a 237–256 peptide [Bob1 (C20) from Santa Cruz], and biotinylated using Sulfo-NHS-LC-Biotin (Pierce). The biotinylated antibodies were revealed with avidin/alkaline phosphatase reagent and an AP-III substrate kit (blue, Vector). Identical GC-specific staining patterns were obtained

with both anti-OCA-B antibodies. Normal rabbit sera as well as purified rabbit IgGs were used as controls.

Antibody assays

Anti-TNP antibody levels were measured by enzyme-linked immunosorbent assay (ELISA). Titers of total mouse Ig as well as the isotype-specific anti-TNP Igs were determined using plates coated with TNP-conjugated bovine serum albumin (TNP-BSA), and biotinylated affinity-purified goat anti-mouse IgM, or goat anti-mouse IgG (Southern Biotech) and peroxidase-conjugated streptavidin (Vector) as the detecting reagents. Results are expressed as OD_{405} absorbance values.

Cell purification

Splenocytes from female C57BL/6 mice (6–9 weeks old, Jackson Lab.) were depleted of red blood cells and subjected to 50–60–66–70% discontinuous Percoll gradient centrifugation. High density cells were collected at the interface of the 66–70% layers and low density cells were pooled from 50–60% and 60–66% layer fractions. T cells were removed by incubating with anti-mouse Thy1.2-coated magnetic beads [Mouse pan-T (Thy1.2), Dynal]. FACS analysis with B220 and IgM, or CD3 staining, showed that the high and low density B cell preparations were 90–93% pure. For T cell purification, unfractionated splenocytes were incubated directly with anti-mouse B220-coated magnetic beads [Mouse pan-B (B220), Dynal].

Bone marrow cells from female C57BL/6 mice (4–5 weeks old, Jackson Lab.) were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgM (R6-60.2, Pharmingen) and phycoerythrin (PE)-conjugated anti-mouse B220 (RA3-6B2, Pharmingen). Large $B220^{+}/IgM^{-}$ and small $B220^{+}IgM^{+}$ B cells were sorted using a FACSVantage (Becton Dickinson).

Tissue culture

Purified high density resting B cells ($2–3\times10^5$/ml) were cultured with RPMI 1640 (Gibco-BRL) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 0.1 mM MEM AA, 1 mM Na pyruvate, 10 mM HEPES 7.2 and 50 μM β-mercaptoethanol (Gibco-BRL). CD40L L cells were used as the source of CD40 ligand, and were maintained in the same medium supplemented with G418 (1 mg/ml) (Gibco-BRL) and 1× HAT (Sigma) (Stuber *et al*., 1995). CD40L stimulation was performed as previously described (Stuber *et al*., 1995). Other reagents used for stimulation experiments were: LPS (*Escherichia coli* 0111:B4, Difco), 25 μg/ml; anti-μ, AffiniPure $F(ab')_2$ fragment of goat anti-mouse IgM (μ) (Jackson ImmunoResearch) 5 μg/ml; IL 4, mouse recombinant (Gibco-BRL), 5 ng/ml (50 U/ml); and IL-7, mouse recombinant (Genzyme), 15 ng (45 U)/ml. ts-Abl pre-B cell line 103/4 was grown in the RPMI 1640 medium with 1 mg/ml G418 (Gibco-BRL) at 34°C, 6% CO_2 as the permissive condition and at 39.5°C, 6% CO_2 as the non-permissive condition (Chen *et al*., 1994).

Protein and RNA analyses

For cells purified by cell sorting, SDS whole-cell lysates were used for Western blot analysis. The lysates were made by sonicating the cell pellet directly into 2× SDS sample buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 1.14 M β-mercaptoethanol, 20% sucrose, 0.05% bromophenol blue) at 1×10^6 cells/100 μl. Otherwise, Western blot analysis was performed on whole-cell extracts, which were made by multiple rounds of freeze–thaw of cells resuspended (at 2×10^7 cells/100 μl) in 20 mM HEPES pH 7.9, 20% glycerol, 400 mM NaCl, 0.5 mM EDTA, 0.25 mM EGTA, 0.025% NP-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM NaF, 0.5 mM Na_3VO_4, 4 μg/ml leupeptin, 4 μg/ml antipain and 1% aprotinin. Protein concentrations were determined with Bradford reagent (Bio-Rad) using BSA as a standard. Proteins were resolved on a 10% SDS–polyacrylamide gel, and transferred onto an Immobilon PVDF membrane (Millipore). The rabbit anti-OCA-B antisera (Luo and Roeder, 1995), and affinity-purified rabbit polyclonal anti-Oct 1 (C-21, Santa Cruz), anti-Oct2 (C-20, Santa Cruz), anti-Bcl-3 (C-14, Santa Cruz), anti-Bcl-6 (C-19, Santa Cruz) and anti-β-actin (A2066, Sigma) antibodies were used for Western blot detection. The antigen-bound rabbit IgGs were visualized with a donkey anti-rabbit/horseradish peroxidase ECL system (Amersham) (Qin *et al*., 1994). RNA preparation and Northern blotting were performed using a mouse full-length OCA-B cDNA as probe (Qin *et al*., 1994; Kim *et al*., 1996).

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