Signaling pathways involved in B cell differentiation and disease: the role of Ral, Btk and Met

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The B Cell Antigen Receptor Controls AP-1 and NFAT Activity through Ras-Mediated Activation of Ral

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Chapter 3
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The B Cell Antigen Receptor Controls AP-1 and NFAT Activity through Ras-Mediated Activation of Ral

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Signaling by the BCR involves activation of several members of the Ras superfamily of small GTPases, among which is Ras itself. Ras can control the activity of multiple effectors, including Raf, PI3K, and guanine nucleotide exchange factors for the small GTPase Ral. Ras, Raf, and PI3K have been implicated in a variety of processes underlying B cell development, differentiation, and function; however, the role of Ral in B lymphocytes remains to be established. In this study, we show that Ral is activated upon BCR stimulation in human tonsillar and mouse splenic B lymphocytes and in B cell lines. Using signaling molecule-deficient B cells, we demonstrate that this activation is mediated by Lyn and Syk, Btk, phospholipase C-γ2, and inositol-1,4,5-trisphosphate receptor-mediated Ca²⁺ release. In addition, although Ral can be activated by Ras-independent mechanisms, we demonstrate that BCR-controlled activation of Ral is dependent on Ras. By means of expression of the dominant-negative mutants RasN17 and RalN28, or of RalBP2GAP, a Ral effector mutant which sequesters active Ral, we show that Ras and Ral mediate BCR-controlled transcription of c-fos. Furthermore, while not involved in NF-κB activation, Ras and Ral mediate BCR-controlled activation of JUN/ATF2 and NFAT transcription factors. Taken together, our data show that Ral is activated upon BCR stimulation and mediates BCR-controlled activation of AP-1 and NFAT transcription factors. These findings suggest that Ral plays an important role in B cell development and function. The Journal of Immunology, 2007, 178: 1405–1414.

The BCR plays an essential role in B cell development, differentiation, and function by controlling adhesion, survival, and proliferation (1, 2). Many BCR-controlled responses depend on activation of the small GTPase Ras (3–11). Expression of RasN17, a dominant-negative Ras mutant, arrests B cell development at a very early stage (6, 9), whereas introduction of the constitutive-active RasV12 mutant into a Ragnull background induces the transition of pro-B cells to pre-B cells and the subsequent generation of mature B cells (7, 8). Furthermore, BCR-controlled proliferation depends on activation of Ras (10) and RasN17 expression impairs recruitment of high-affinity precursors into the memory B cell compartment and prevents terminal differentiation of IgG memory B cells in response to recall Ag (11).

The Ras GTPase exerts a wide variety of biological effects by controlling multiple effectors, including Raf (12), PI3K (13), and guanine nucleotide exchange factors (GEFs) (14) for the small GTPase Ral (14, 15). Raf and PI3K function downstream of the BCR and are critically involved in B cell development, differentiation, and function. Raf phosphorylates and activates the protein kinase MEK, which in turn phosphorylates and activates the MAPKs ERK 1 and 2. Expression of an activated form of Raf partially rescues a RasN17-induced block in B cell development (6) and activation of the Raf-MEK-ERK pathway is required for a subset of B cell Ag responses (16, 17). PI3K controls the activity of a variety of signaling molecules, including protein kinase B (PKB)/Akt, the Tec-kinase family member Bruton’s tyrosine kinase (Btk) and Rac (1, 18). Mice deficient in specific isoforms of PI3K reveal an essential role for PI3K in B cell development and distinct B cell responses (18). Thus far, however, no data are available concerning the role in BCR signaling of the third group of Ras effectors, i.e., the GEFs that activate Ral.

Ral has been implicated in a wide variety of cellular responses, like cytoskeletal rearrangements, migration, endo- and exocytosis, proliferation (reviewed in Ref. 19), and accumulating evidence indicates that Ral is an essential mediator of Ras-induced tumorigenesis (20–23). Ras can bind to and regulate the activity of phospholipase D1 (24), the Sec5 and Exo84 subunits of the exocyst complex (25, 26), the actin-binding protein filamin (27), and the Cdc42/Rac GTPase-activating protein (GAP) Ral-binding protein (RalBP)-1 (28, 29). In addition, the RalGEF-Ral pathway can regulate gene transcription by modulating the activity of various transcription factors. For example, Ral is involved in the transcriptional activation of the c-fos promoter (30–32) and mediates Ras-controlled phosphorylation of c-Jun (33) and ATF2 (34). Furthermore, Ral signaling modulates the transcriptional activity of the FOXO family member AFX (35–37), mediates efferal growth factor-induced Stat3 activation via Src (38), and regulates the relief of transcriptional repression by ZO-1-associated nucleic acid-binding protein (39). In addition, in fibroblasts, Ral is able to activate NF-κB, resulting in an increase of cyclin D1 expression (40). In this study, we examined whether and how Ral is activated upon BCR stimulation. Indeed, Ral was found to be activated upon BCR stimulation, which is mediated by Lyn and Syk, Btk, phospholipase C-γ2 (PLCy2), the inositol-1,4,5-trisphosphate receptor (IP₃R),-mediated Ca²⁺ release, and Ras. Furthermore, we demonstrate that Ral mediates BCR-induced activation of the AP-1 and NFAT transcription factors, thus establishing an important role for Ral in BCR-controlled gene transcription.
Materials and Methods

Antibodies
Mouse mAbs used were: anti-RalA (IgG2a), anti-Ras (BD Biosciences). Polyclonal Abs used were: goat anti-chicken IgM (Bethyl Laboratories), mouse anti-human IgM (MH15; provided by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), F(ab')2 of rabbit anti-human IgM (DakoCytomation), F(ab')2 of goat anti-mouse IgM (Jackson ImmunoResearch Laboratories), rabbit anti-phospho-PKB/Akt (Ser473), rabbit anti-phospho-p44/42 MAPK (Thr202/Tyr204) (both New England Biolabs); HRP-conjugated rabbit anti-mouse and HRP-conjugated goat anti-rabbit (both DakoCytomation).

Plasmids
pA-purol–RasN17 (41) was provided by Dr. T. Kurosaki (Kansai Medical University, Moriyuji, Japan). The 6xNFkB-RE- and 4xFAT-RE-luciferase reporter constructs were obtained from Stratagene, pRK5-RalBP1AGAP, pMT2-HA-RalN28, pMT2-HA-Rlf-CAAX, and the c-fos-luciferase, tata-luciferase, and 5xjun2-tata-luciferase reporters were described previously (30, 33, 34). 6xNFkB-RE-luciferase reporter and pMT2-HA-Rlf-CAAX were provided by Dr. K. Reedquist (Academic Medical Center (AMC), Amsterdam, The Netherlands). pRK5-RalBP1AGAP was provided by Dr. F. Zwartkruis (Utrecht Medical Center, Utrecht, The Netherlands). 5xjun2-tata-luciferase (34), tata-luciferase control, and c-fos-luciferase (42) reporters were provided by Dr. H. van Dam (Leiden University Medical Centre, Leiden, The Netherlands). 4xFAT-RE-luciferase reporter was provided by Dr. P. Schrier (Leiden University Medical Centre, Leiden, The Netherlands).

Isolation of tonsillar B cells and murine splenic B cells
Human tonsillar B cells were isolated essentially as described previously (2). All procedures were done following a protocol agreed upon by the AMC Medical Ethical Committee. Mouse splenic B cells were obtained from C57BL/6 mice, bred and maintained at the animal care facility of the AMC according to institutional and national guidelines. The splenic B cells were isolated using the MACS system (Miltenyi Biotec) by positive selection with anti-CD45R (B220) microbeads, essentially according to manufacturer’s instruction. Isolated B cells were maintained in RPMI 1640 containing 10% FCS and were used immediately.

Cell lines
The Burkitt’s lymphoma cell line Ramos was cultured in Iscove’s medium (Invitrogen Life Technologies) containing 10% fetal clone I serum (HyClone), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Invitrogen Life Technologies), 20 mg/ml human recombinant transferrin (Sigma-Aldrich), 50 mM 2-ME. The chicken bursal lymphoma cell line DT40 and DT40 cells deficient in both Lyn and Syk, Btk, PLCγ2, or for all three types of PLCs; cells obtained from RIKEN Cell Bank (Tsukuba Science City, Japan) with permission from Dr. T. Kurosaki, were cultured at 39.5°C as described (41). All DT40 cells showed similar expression of surface IgM as determined by FACS analysis using goat anti-chicken IgM (10 μg/ml).

GTPase pull-down assays
Cells were resuspended in RPMI 1640 to 2.0 × 10⁷ cells/ml and stimulated with 10 μg/ml anti-IgM or F(ab’)2 of anti-IgM (primary B cells), 50 ng/ml PMA, or 1 μM ionomycin, as indicated. Reactions were terminated by adding an equal volume of cold 2× lysis buffer (100 mM Tris-HCl (pH 7.4), 400 mM NaCl, 5 mM MgCl2, 2% Nonidet P-40, 2× EDTA-free protease mixture tablets (Roche) per 50 ml). After 10 min on ice, cell debris was removed by centrifugation. Cell lysates were used immediately for GTPase pull-down assays. For this purpose, glutathione-Sepharose beads (10 μl of a 2% solution per sample) were precoupled with GST-RalBP1-Ral-binding domain (RBD) or GST-Ral-Ras-binding domain fusion protein by continuous mixing for 30 min at 4°C with bacterial cell lysates from Escherichia coli strain AD202 transformed with pGEX4T3-RalBP-RBD (43) or E. coli strain BL21 transformed with pGEX2T-Ral-RBD (44), respectively. After being washed three times with lysis buffer, these precoupled beads were added to the cell lysates, and incubated for 30 min at 4°C during continuous mixing. Finally, the beads were washed four times with lysis buffer, bound proteins were eluted with sample buffer, separated by 12 or 15% SDS-PAGE, and immunoblotted with anti-RalA or anti-Ras.

Generation of stably transfected DT40 cells
A total of 25 μg of linearized pA-purol–RasN17 was mixed with 10⁷ DT40 B cells in 0.5 ml of RPMI 1640 medium in a 0.4-cm electrode gap and electroporated using a Gene Pulser Apparatus (Bio-Rad) and electroporated using a Gene Pulser Apparatus with Capacitance Extender (Bio-Rad) at 250 V, 960 μF. After 24 h recovery at 39.5°C in DT40 medium, cells were selected in DT40 medium containing 0.5 μg/ml Puromycin (Sigma-Aldrich). Puromycin-resistant clones were screened for expression of RasN17 by immunoblotting.

Immunoblotting
Immunoblotting was performed essentially as previously described (2). Quantification was performed using Image-Pro plus (MediaCybernetics) software.

Transfections and luciferase assays
A total of 10⁷ DT40 B cells in 0.5 ml of RPMI 1640 medium were transfected by electroporation as indicated above with 5 μg of firefly luciferase reporter construct, 1 μg of pRL-TK (Promega), together with the indicated expression plasmids or empty control plasmid up to a total amount 30 μg of DNA per transfection. Cells transfected with c-fos-, tata-, 5xjun2-tata-, or 4xFAT-RE-luciferase reporter constructs were allowed to recover for 8 h at 39.5°C in DT40 B cell medium and subsequently serum starved for 16 h. Cells transfected with 6xNFkB-RE-luciferase reporter construct were allowed to recover for 16 h at 39.5°C in DT40 B cell medium and were not serum starved. Finally, the cells were resuspended in RPMI 1640 medium (3–8 × 10⁶ viable cells/ml) and incubated in the presence of 10 μM anti-IgM, 50 ng/m 1l PMA, or 1 μM ionomycin, as indicated, at 39.5°C for 8 h. Lysis and determination of luciferase activity was conducted according to manufacturer’s instructions (Dual-Luciferase Reporter Assay System; Promega), using Rentilla luciferase activity as an internal control.

Statistical analysis
The unpaired two-tailed Student’s t test was used to determine the significance of differences between means. All relevant comparisons (e.g., control vs dominant-negative mutant-transfected cells or anti-IgM-stimulated wild-type (WT) vs gene-deficient DT40 cells) were significantly different (p < 0.05), unless otherwise indicated.

Results
Ral is activated upon BCR activation
To investigate whether stimulation of the BCR induces activation of the Ral GTPase, we performed Ral pull-down assays. Ral proteins in their active GTP-bound conformation have high affinity for the RBD of RalBP1, whereas Ral proteins in their inactive GDP-bound conformation do not. By using a fusion protein consisting of the RalBP-RBD fused to GST, active Ral proteins can be precipitated from cell lysates and be monitored by immunoblotting with anti-Ral Abs (43).

Using this assay, we found that cross-linking of the BCR with anti-IgM resulted in an increase of active Ral in human tonsillar B cells (Fig. 1). Elevated levels of Ral-GTP were already observed upon BCR stimulation with anti-IgM, as determined by FACS analysis using goat anti-chicken IgM (10 μg/ml).

BCR-controlled activation of Ral is mediated by Lyn/Syk, Btk, PLCγ2, and IP3, Rs
The most receptor-proximal events upon BCR ligation by Ag are the activation of the Src-family protein tyrosine kinase Lyn, and the protein tyrosine kinase Syk. To determine whether these signaling molecules are involved in BCR-controlled Ral activation, pull-down assays were performed using DT40 cells deficient in both Lyn and Syk. In these cells, no Ral activation could be observed upon BCR activation, indicating that Lyn, Syk, or both are required for BCR-controlled activation of Ral (Fig. 2A).

Lyn and Syk mediate the phosphorylation and activation of the Tec kinase family protein tyrosine kinase Btk, which is critically involved in several BCR-controlled cellular responses (1). In DT40 cells deficient in Btk, only a partial activation of Ral was
observed after BCR stimulation, demonstrating Btk is required for full Ral activation by the BCR (Fig. 2B).

PLCγ2 is a substrate for Btk that mediates various BCR-controlled cellular responses (45). For example, BCR-controlled ERK activation is partly dependent on PLCγ2 (41) and Ras activation in response to BCR triggering is severely reduced in DT40 cells deficient in PLCγ2 (46) (see also Fig. 3A). To investigate whether PLCγ2 is also involved in BCR-controlled Ral activation, PLCγ2-deficient DT40 cells were used. In these cells, Ral activation upon BCR ligation was reduced compared with the activation observed in DT40 WT cells (Fig. 2C). Notably, stimulation of DT40 cells with PMA, which mimics the actions of diacylglycerol (DAG), one of the second messengers produced by PLC activity, was not sufficient for activation of Ral (Fig. 2E). Besides forming DAG, PLCγ2 activation leads to production of IP3, which results in IP3R-mediated release of Ca2+ from intracellular stores. In DT40 cells deficient in all three expressed IP3R isoforms, activation of Ral upon BCR stimulation is impaired (Fig. 2D). Furthermore,

**FIGURE 1.** BCR stimulation induces activation of Ral. Tonsillar and splenic B cells, and Ramos and DT40 B cell lines, were stimulated for the indicated periods of time with anti-IgM Abs (aIgM) or F(ab’)2 thereof (tonsillar and splenic B cells), lysed, and the amounts of Ral-GTP in the lysates were determined by pull-down assay using GST-RalBP-RBD fusion protein (PD). As a control, total lysate (TL) proteins were immunoblotted and probed using anti-RalA Abs (+, Ig L chain). The results are representative of at least two independent experiments.

**FIGURE 2.** BCR-induced activation of Ral is mediated by Lyn/Syk, Btk, PLCγ2, and IP3Rs. WT (A–E) and Lyn/Syk double-deficient (A), Btk- (B), PLCγ2- (C), or IP3R-deficient DT40 cells (D) were stimulated for the indicated period of time with either anti-IgM (aIgM) Abs, PMA, ionomycin, or PMA and ionomycin, as indicated, lysed, and the amounts of Ral-GTP in the lysates were determined by pull-down assay using GST-RalBP-RBD fusion protein (PD). As a control, total lysate (TL) proteins were immunoblotted and probed using anti-RalA Abs. The results are representative of at least two independent experiments.
whereas stimulation with PMA or the Ca\(^{2+}\)-ionophore ionomycin alone had no effect on Ral activation, PMA combined with ionomycin did induce activation of Ral (Fig. 2E). Taken together, these results imply that BCR-controlled activation of Ral involves activation of PLC\(\gamma\)-2 and the subsequent IP\(_3/R\)-mediated release of Ca\(^{2+}\) from intracellular stores.

**BCR-controlled activation of Ral requires Ras activation**

Activation of Ral can occur via Ca\(^{2+}\) (43) and calmodulin (47), \(\beta\)-arrestins (48), or Ras (14, 15). Similar to Ras, and supporting a role for Ras in activation of Ral by the BCR, BCR-controlled activation of Ras and Ras-mediated phosphorylation of ERK is impaired in PLC\(\gamma\)-2-deficient DT40 cells (46) (Fig. 3A). In contrast to Ras, however, BCR-controlled activation of Ras and phosphorylation of ERK is not affected in IP\(_3/R\)-deficient DT40 cells (Fig. 3A). To investigate directly whether activation of Ral by the BCR is Ras dependent or can also be activated in a Ras-independent manner, we generated DT40 cells stably expressing RasN17, a dominant-negative mutant. In these DT40 RasN17 cells, activation of Ral upon BCR stimulation was completely abolished (Fig. 3B). Thus, these data demonstrate a critical requirement for Ras in activation of Ral by the BCR.

**Ral mediates BCR-controlled transcriptional activation of the c-fos promoter**

In fibroblasts and neural cells, Ral has been shown to control expression of the c-fos proto-oncogene (30, 49). Furthermore, stimulation of the BCR results in c-fos transcription (50). To examine the signaling mechanism underlying BCR-controlled transcription of c-fos, DT40 cells were transfected with a reporter construct containing a luciferase reporter gene under transcriptional control of the c-fos promoter. As shown in Fig. 4A, BCR ligation resulted in an increase of reporter activity in a Btk- and PLC\(\gamma\)-2-dependent manner. This PLC\(\gamma\)-2 dependency appears to rely mainly on the formation of DAG, because PMA stimulation induced full reporter activation, whereas ionomycin, which induces an increase of intracellular Ca\(^{2+}\) levels, had no effect on reporter activity (Fig. 4A). In addition, BCR-controlled reporter activity was not affected in cells deficient in IP\(_3/Rs\) (Fig. 4A). Furthermore, transient expression of RasN17 reduced BCR-controlled transcriptional activation of the c-fos promoter (by \(-50\%\); Fig. 4B). Similar results were obtained using DT40 cells stably expressing RasN17 (data not shown). To examine the role of Ral in this response, we used a dominant-negative mutant of this GTPase, RasN17, and RasBP\(_{1}\)GAP, a RasBP1 protein lacking the GAP domain which sequesters active Ral proteins, and thereby inhibits binding to endogenous effector proteins (33). Both resulted in a reduction of BCR-controlled c-fos-reporter activity (by \(-25\%\) and \(-50\%\) respectively; Fig. 4, C and D). Conversely, transfection of the c-fos-luciferase reporter together with constructs expressing mutant proteins which activate Ral, like the constitutively active RalGGEF Rif-CAAX, or RasV12G37, a constitutively active Ras mutant which specifically activates Ral signaling, did not result in enhanced reporter activity (data not shown). Taken together, these data show Ras and Ral mediate expression of fos in response to BCR activation, but activation of Ral by itself is not sufficient to induce expression of this AP-1 family member in B cells.

**Ral mediates BCR-controlled JUN/ATF2 activity**

Besides regulating expression of fos, Ral has also been found to control the activity of JUN and ATF2, via JNK and p38, respectively (33, 34). Activation of JNK, p38, and these transcription factors has also been observed upon BCR stimulation (41, 50–52). By transiently transfecting the signaling molecule-deficient DT40 cells with either the JUN/ATF2-dependent luciferase reporter 5xjun2-tata-luciferase (which contains JUN/ATF2-responsive elements originating from the c-jun promoter) or the tata-luciferase control, we found that activation of the BCR resulted in enhanced JUN/ATF2
transcriptional activity in DT40 WT cells, but not in cells deficient in Btk, PLCγ2, or IP3Rs (Fig. 5A). BCR stimulation had no effect on the reporter activity of the tata-luciferase control (data not shown). Expression of RasN17, by means of transient transfection or using DT40 cells stably expressing RasN17, resulted in severely reduced reporter activity (by ~45%; Fig. 5B and data not shown). To investigate whether Ral mediates BCR-controlled transcriptional activation of JUN and/or ATF2, DT40 cells were transiently transfected with 5xjun2-tata-luciferase together with either RalN28 (Fig. 5C) or RalBPΔGAP (Fig. 5D). Inhibition of Ral activity clearly reduced BCR-controlled transcriptional activity on the 5xjun2-tata-luciferase reporter (by ~30–35%; Fig. 5, C and D). Thus, our data indicate that Ras and Ral are required for optimal BCR-controlled activation of JUN/ATF2-mediated transcription.

Ral mediates BCR-controlled activity of NFAT, but not of NF-κB
NF-κB and NFAT transcription factors play an essential role in B cell development and differentiation (53, 54). BCR stimulation has been
shown to result in activation of these proteins (55–57), and in fibroblasts, Ral has been found to activate NF-κB, leading to increased cyclin D1 expression (40). To study the role of Ral in NF-κB activation by the BCR, DT40 cells were transfected with a construct containing the luciferase reporter gene under transcriptional control of multimerized NF-κB responsive elements (6xNF-κB-RE-luciferase). As reported previously (58–61), NF-κB activity was found to depend on Btk and PLCγ2 (Fig. 6A). Furthermore, BCR-controlled NF-κB activity was severely reduced in cells deficient in IP3Rs (Fig. 6A), demonstrating the involvement of the IP3-mediated Ca2+ response. In contrast, inhibition of Ras or Ral, by means of the stable expression of RasN17 (data not shown), or the transient expression of RasN17 (Fig. 6B), RalN28 (Fig. 6C), or RalBP1GAP (Fig. 6D), did not affect BCR-induced NF-κB activation. Thus, although Ral has been shown to regulate NF-κB transcriptional activity in fibroblasts (40), in B cells, activation of NF-κB by the BCR is independent of Ras and Ral. This shows that the function of Ral in controlling gene transcription varies depending on the cell type and the signaling route.

**FIGURE 6.** BCR activation induces NF-κB activity through Btk, PLCγ2, and IP3Rs, but not through Ras and Ral. A, WT, Btk-, PLCγ2-, and IP3R-deficient DT40 cells transiently transfected with a 6xNFκB-RE-luciferase reporter construct were stimulated for 8 h with anti-IgM (αIgM), PMA, or ionomycin, lysed, and luciferase activity was determined. B–D, DT40 cells were cotransfected with a 6xNFκB-RE-luciferase reporter and RasN17 (B), RalN28 (C), RalBP1GAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (αIgM), lysed, and luciferase activity was determined. A–D, Normalized reporter activity is shown as the mean ± SD of triplicates. Comparisons of anti-IgM-stimulated WT vs gene-deficient DT40 cells were significantly different (p < 0.005). The results are representative of at least three independent experiments.

**FIGURE 7.** BCR activation induces NFAT activity through Btk, PLCγ2, IP3Rs, Ras, and Btk. A, WT, Btk-, PLCγ2-, and IP3R-deficient DT40 cells transiently transfected with a 4xNFAT-RE-luciferase reporter construct were stimulated for 8 h with anti-IgM (αIgM), PMA or ionomycin, lysed, and luciferase activity was determined. B–D, DT40 cells were cotransfected with a 4xNFAT-RE-luciferase reporter and RasN17 (B), RalN28 (C), RalBP1GAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (αIgM), lysed, and luciferase activity was determined. A–D, Normalized reporter activity is shown as the mean ± SD of triplicates. All relevant comparisons (e.g., control vs dominant-negative mutant transfected cells or anti-IgM-stimulated WT vs gene-deficient DT40 cells) were significantly different (p < 0.05). The results are representative of at least three independent experiments.
As mentioned, BCR stimulation results in activation of NFAT (56, 57). Transcription of many NFAT target genes depends on the interaction between NFAT and AP-1 on composite promoter regions of these genes (62). By transfecting DT40 cells with a construct containing the luciferase reporter gene under transcriptional control of multimerized NFAT responsive elements (4xNFAT-RE-luciferase), we found that BCR-controlled NFAT activity requires Btk, PLC\_\gamma2, the IP3-mediated Ca\textsuperscript{2+} release and Ras activity (Fig. 7, A and B). Similar results were previously reported by Hao et al. (63) and Antony et al. (61). Besides Ca\textsuperscript{2+} release, also generation of DAG by PLC is required for NFAT activation, because only ionomycin combined with PMA resulted in maximal NFAT activity (Fig. 7A). Moreover, we observed that BCR-controlled NFAT activity is inhibited by cotransfection of RasN17 (by ~25%; Fig. 7B) or RaIIP\_\gammaGAP (by ~40%; Fig. 7D), indicating that Ras mediates Ras-controlled activation of NFAT in response to BCR stimulation.

**Discussion**

**BCR-controlled activation of Ras is mediated by Lyn/Syk, Btk, PLC\_\gamma2, IP3-mediated Ca\textsuperscript{2+} release, and Ras**

In this study, we have shown that BCR stimulation of primary B cells and B cell lines results in activation of Ras. BCR-controlled activation of Ras was completely absent in DT40 cells deficient in both Lyn and Syk, demonstrating the requirement for one or both of these cytoplasmic kinases in the underlying signaling mechanism (Fig. 2A). In BCR signaling, the Tec kinase family member Btk functions downstream of these kinases. Mutations in Btk cause the immunodeficiency disease X-linked agammaglobulinemia in humans and Xid in mice, due to a severe reduction of mature B cell numbers and decreased Ig serum levels. Btk was found to be involved in BCR-controlled activation of Ras, because Ras activation was reduced in DT40 cells deficient in Btk (Fig. 2A). Btk mediates BCR-controlled activation of PLC\_\gamma2 (45), which leads to the generation of DAG and IP3. The latter binds IP3Rs and induces the release of Ca\textsuperscript{2+} from intracellular stores. Like Btk, PLC\_\gamma2 was found to be involved in activation of Ras, and because Ras activation was reduced in cells deficient for IP3Rs, this activation involves the PLC\_\gamma2-controlled increase of intracellular Ca\textsuperscript{2+} (Fig. 2, C and D). Furthermore, stimulation of DT40 B cells with PMA, which mimics the actions of DAG, did not result in Ras activation (Fig. 2E), indicating that the BCR-controlled production of DAG alone is not sufficient for activation of Ras. Notably, some activation of Ras still remains upon BCR stimulation of B cells deficient in Btk, PLC\_\gamma2, and IP3Rs, demonstrating the presence of a Lyn/Syk-dependent mechanism of activation, independent of these signaling molecules, as well (Fig. 8).

We found that expression of the dominant-negative mutant RasN17 abolished activation of Ras upon BCR stimulation (Fig. 3B), thereby demonstrating that Ras mediates activation of Ras by the BCR. The activation of Ras is impaired in PLC\_\gamma2-deficient
cells, but not in IP<sub>R</sub>-deficient cells (Fig. 3A), indicating the involvement of DAG production rather than Ca<sup>2+</sup> release in Ras activation. Indeed, the generation of DAG upon BCR stimulation can recruit the Ras exchange factor RasGRP3 to the membrane, where it is subsequently phosphorylated and activated by the action of protein kinase C, resulting in activation of Ras (64, 65). The residual Ras activation that we observed in PLCγ2-deficient cells most likely involves Son-of-Sevenless (SOS) activity (66). Interestingly, the activation of Ras is impaired in PLCγ2- as well as IP<sub>R</sub>-deficient cells (Fig. 2, C and D). Besides the critical role for Ras in Ral activation, this also points toward an important role for Ral in a Ca<sup>2+</sup>-dependent signaling molecule (Fig. 8). A likely candidate is calmodulin, which has been shown to interact with Ral in a Ca<sup>2+</sup>-controlled manner, resulting in an increase of active Ral (47, 67).

**Ral mediates BCR-controlled gene transcription**

To study the possible role of Ral in BCR-controlled gene transcription, we made use of the dominant-negative RalN28 mutant and the mutant Ral effector RalBP<sub>GAP</sub>. The RalN28 protein is unable to bind guanine nucleotides and exerts its inhibitory effect by binding RalGEFs which are thereby unable to catalyze the exchange of guanine nucleotides from endogenous Ras. However, similar to the Rap1 exchange factor Epac1, RalGEFs may also transduce signals in a GTPase-independent manner (68). This alternative pathway would be inhibited by expression of RalN28 as a consequence of steric hindrance or conformational changes induced by the binding of RalN28 to RalGEFs. Therefore, to exclude a consequence of steric hindrance or conformational changes of guanine nucleotides from endogenous Ras. However, residual Ras activation that we observed in PLCγ2-deficient cells (Fig. 2A), indicating the important role of Ral in Ca<sup>2+</sup>-dependent signaling molecule (Fig. 8). A likely candidate is calmodulin, which has been shown to interact with Ral in a Ca<sup>2+</sup>-controlled manner, resulting in an increase of active Ral (47, 67).

One gene whose expression and function has previously been shown to be regulated by Ral is the c-fos proto-oncogene (30–32), which also acts downstream of the BCR (50). By expressing the dominant-negative RalN28 mutant or the mutant Ral effector RalBP<sub>GAP</sub> we have demonstrated that Ral is involved in BCR-controlled transactivation of the c-fos promoter (Fig. 4, C and D). Although c-fos-luciferase reporter activity was not affected in cells deficient in IP<sub>R</sub>,R<sub>s</sub> (Fig. 4A), it was impaired in cells transfected with a dominant-negative Ras (Fig. 4B), suggesting that Ras rather than Ca<sup>2+</sup>-controlled Ras activity is responsible for the BCR-induced FOS expression.

A major regulator of c-fos transcription is the serum response factor, a downstream target of the Ras-ERK pathway. BCR activation controls serum response factor activity through Lyn, Syk, Btk, PLCγ2, Ca<sup>2+</sup>, and Ras (63), which we also found to mediate Ral activation (Figs. 2 and 3). Furthermore, FOS expression is controlled by the transcription factor ATF2. Because Ral mediates BCR-controlled activation of ATF2-dependent gene expression (Fig. 5, C and D), Ral may mediate expression of FOS through ATF2. Indeed, Ouwens et al. (34) showed that growth factor-induced activation of ATF2 involves its phosphorylation via the Ras-RalGDS-Ral-p38 MAPK pathway. Activation of the p38 MAPK in response to BCR stimulation was found to be independent of Btk, but was abolished in cells deficient for both Lyn and Syk (52). Because partial Ral activation upon BCR stimulation could still be observed in Btk-deficient cells (Fig. 2B), but not in Lyn/Syk double-deficient cells (Fig. 2A), BCR-controlled p38 activation may be mediated by Ral. Furthermore, Ral has also been shown to control JNK activity, which mediates phosphorylation of c-Jun (33). BCR-controlled activation of JNK is totally dependent on Btk and PLCγ2, and partially on Ras (41, 52). Combined with our results showing that Ral is involved in activation of the JUN/ATF2-dependent luciferase reporter (Fig. 5), this suggests that Ral is involved in BCR-controlled regulation of JNK activity and JUN-mediated gene transcription. Taken together, Ral may mediate BCR-controlled p38 and JNK activation, which in turn results in phosphorylation and activation of ATF2 and c-Jun respectively, and consequently leads to expression of FOS (Fig. 8). The notion that transcriptional activity of ATF2 requires phosphorylation via more than just one signal transduction pathway (34) is in accordance with our observation that activation of Ral alone is not sufficient to induce FOS expression.

The FOS and JUN proteins form dimeric complexes that stimulate transcription of genes containing AP-1 regulatory elements, thereby controlling proliferation, apoptosis, transformation, differentiation, and development (69, 70). In mouse B cells, overexpression of FOS has been described to inhibit cell cycle progression (71) and to induce apoptosis (72), thus emphasizing the importance of tight regulation of FOS expression in B cells. Moreover, recently a role for FOS in the regulation of Blimp-1 expression and terminal differentiation of activated B cells has been described (73). The functional consequence of AP-1 activity is often determined by the interaction with other transcription factors. Examples are NFAT proteins (62), which control proliferation, apoptosis, and cytokine production (74, 75). Because FOS is part of the AP-1 complex involved in NFAT DNA binding in B cells (56, 76), one could argue that NFAT-mediated transcription may be regulated by Ral. Indeed, we have demonstrated that Ral is involved in controlling BCR-controlled NFAT transcriptional activity (Figs. 7, C and D, and 8). In B cells, NFAT1 (or NFATc2) and NFAT2 (or NFATc1) have been found to control expression of TNF-α (77), Igα (76), and CD5 (78). In addition, NFAT2 is required for development of B-1a cells (79), which plays an important role in T cell-independent Ag responses. Furthermore, mice repopulated with B cells deficient for both NFAT1 and NFAT2 showed increased levels of IgG1 and IgE and expanded populations of plasma cells (54), indicating NFAT proteins are involved in both normal B cell homeostasis and differentiation. Thus, because Ral has been implicated in activation of NFAT proteins, Ral may be involved in controlling these processes.

Several lines of evidence suggest that, similar to nonmalignant B cells, selection for expression of a functional BCR also occurs in certain B cell lymphomas, including chronic lymphatic leukemias, follicular, Burkitt’s, and mucosa-associated lymphoid tissue lymphomas (80, 81). The signals supplied by the BCR, including activation of Ras and Ral, may promote growth and survival of B cell lymphomas. Indeed, whereas oncogenic Ras mutations have been detected in 40–50% of patients with multiple myeloma (82, 83), a neoplasms of terminally differentiated B cells which do not express a functional BCR, no activating Ras mutations have been found in BCR-expressing lymphomas. Thus, because Ras and Ral become activated by BCR signaling, activating Ras mutations may not be required for growth of BCR-expressing lymphomas.

In conclusion, because Ral mediates BCR-controlled activation of the transcription factors AP-1 and NFAT, Ral may play an important role in B cell development and function, and possibly also in the pathogenesis of B cell malignancies.

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Disclosures

The authors have no financial conflict of interest.

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