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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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 Ras. Ras, Raf, and PI3K have been implicated in a variety of processes underlying B cell development, differentiation, and function; however, the role of Raf in B lymphocytes remains to be established. In this study, we show that Raf 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 Raf can be activated by Ras-independent mechanisms, we demonstrate that BCR-controlled activation of Raf is dependent on Ras. By means of expression of the dominant-negative mutants RasN17 and RafN28, or of RafBP2GAP, a Raf effector mutant which sequesters active Raf, we show that Ras and Raf mediate BCR-controlled transcription of c-fos. Furthermore, while not involved in NF-κB activation, Ras and Raf mediate BCR-controlled activation of JUN/ATF2 and NFAT transcription factors. Taken together, our data show that Raf is activated upon BCR stimulation and mediates BCR-controlled activation of AP-1 and NFAT transcription factors. These findings suggest that Raf 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 constitutively active RasV12 mutant into a Rag-null 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 receptors 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 (GEFs3) for the small GTPase Ras (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 Raf.

Raf 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 Raf is an essential mediator of Ras-induced tumorigenesis (20–23). Raf 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) Raf-binding protein (RafBP)-1 (28, 29). In addition, the RafGEF-Raf pathway can regulate gene transcription by modulating the activity of various transcription factors. For example, Raf 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, Raf signaling modulates the transcriptional activity of the FOXO family member AFX (35–37), mediates epidermal 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, Raf is able to activate NF-κB, resulting in an increase of cyclin D1 expression (40). In this study, we examined whether and how Raf is activated upon BCR stimulation. Indeed, Raf was found to be activated upon BCR stimulation, which is mediated by Lyn and Syk, Btk, phospholipase C-γ2 (PLCγ2), the inositol-1,4,5-trisphosphate receptor (IP3;R)-mediated Ca2+ release, and Ras. Furthermore, we demonstrate that Raf mediates BCR-induced activation of the AP-1 and NFAT transcription factors, thus establishing an important role for Raf in BCR-controlled gene transcription.

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3 Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; PKB, protein kinase B; Btk, Bruton’s tyrosine kinase; RafBP, Raf-binding protein; PLC, phospholipase C; IP3;R, inositol-1,4,5-trisphosphate receptor; WT, wild type; RBD, Raf/Ras-binding domain; DAG, diacylglycerol; GAP, GTPase-activating protein.

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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-puroll-RasN17 (41) was provided by Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan). The 6xNFRE-B-RE- and 4xFATRE-luciferase reporter constructs were obtained from Stratagene, pK5-RalBPAGAP, pM2-RAI-RIF-CAAX, and the c-fos-luciferase, tata-luciferase, and 5xJun2-tata-luciferase reporters were described previously (30, 33, 34). 6xNFRE-B-RE-luciferase reporter and pMT2-HA-RIF-CAAX were provided by Dr. K. Reedquist (Academic Medical Center (AMC), Amsterdam, The Netherlands). pK5-RalBPAGAP 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). 4xFATRE-RE-luciferase reporter construct was provided by Dr. P. Schier (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 Technoloiges) containing 10% fetal crude serum (HyClone), 100 µM penicillin, and 100 µM 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 molecules are involved in BCR-controlled Ral activation, pull-down assays were performed using DT40 cells deficient in Lyn and Syk. In these cells, no Ral activation could be observed upon BCR ligation by Ag. 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).

Generation of stably transfected DT40 cells
A total of 25 µg of linearized pA-puroll-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 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.

Statistical analysis
The unpaired two-tailed Student 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 after 2 min of anti-IgM stimulation and persisted for at least 10 min. A similar pattern of Ral activation was observed after BCR activation of mouse splenic B cells, and DT40 and Ramos B cell lines (Fig. 1). These results show that stimulation of the BCR leads to activation of the small GTPase Ral.

BCR-controlled activation of Ral is mediated by Lyn/Syk, Btk, PLCγ2, and IP3Rs
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

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 4xFATRE-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 6xNFRE-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 × 107 viable cells/1.5 ml), and incubated in the presence of 10 µg/ml anti-IgM, 50 ng/ml 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 Renilla luciferase activity as an internal control.

Gene Pulser Cuvette (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.
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,
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), β-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 Ras 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\(_3\)R (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 RalBP1 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 RalGEF RII-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 Ras 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, Ras 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 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 transcription (50). To examine directly whether activation of Ral by the BCR mediates BCR-controlled transcriptional activation of the c-fos promoter, we used a dominant-negative mutant of this GTPase, RasN17, and RalBP1 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 RalGEF RII-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 Ras by itself is not sufficient to induce expression of this AP-1 family member in B cells.

**FIGURE 3.** BCR-controlled Ras activation mediates activation of Ral. A, WT, PLC\(\gamma\)2-, and IP\(_3\)-R-deficient DT40 cells were stimulated for the indicated periods of time with anti-IgM (sIgM) Abs or PMA and ionomycin (P/I), lysed, and the amounts of Ras-GTP in the lysates were determined by pull-down assay using GST-Ral-RBD fusion protein (PD). As a control, total lysate (TL) proteins were immunoblotted and probed using anti-Ras, anti-P-PKB, or anti-P-MAPK (P-ERK) Abs. B, WT and stably transfected DT40 cells were stimulated for the indicated period of time with anti-IgM (sIgM) Abs, lysed, and the amounts of Ras-GTP in the lysates were determined by pull-down assay using GST-RalBP1-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.
transcriptional activity in DT40 WT cells, but not in cells deficient in Btk, PLCζ2, IP, Rs, Ras, and Ral. A. WT, Btk-, PLCζ2-, and IP, R-deficient DT40 cells transiently transfected with a c-fos-luciferase reporter construct were stimulated for 8 h with anti-IgM (rdgM), PMA, or ionomycin, lysed and luciferase activity was determined. B–D. DT40 cells were co-transfected with a c-fos-luciferase reporter and RasN17 (B), RalN28 (C), RalBPΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (rdgM), 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 < 0.01). The results are representative of at least three independent experiments.

BCR activation induces JUN/ATF2 activity through Btk, PLCζ2, or IP, Rs (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.

**Figure 4.** The BCR controls transcription of c-fos through Btk, PLCζ2, IP, Rs, Ras, and Ral. A. WT, Btk-, PLCζ2-, and IP, R-deficient DT40 cells transiently transfected with a c-fos-luciferase reporter construct were stimulated for 8 h with anti-IgM (rdgM), PMA, or ionomycin, lysed and luciferase activity was determined. B–D. DT40 cells were co-transfected with a c-fos-luciferase reporter and RasN17 (B), RalN28 (C), RalBPΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (rdgM), 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 < 0.01). The results are representative of at least three independent experiments.

**Figure 5.** BCR activation induces JUN/ATF2 activity through Btk, PLCζ2, IP, Rs, Ras, and Ral. A. WT, Btk-, PLCζ2-, and IP, R-deficient DT40 cells transiently transfected with the JUN/ATF2-dependent luciferase reporter 5xjun2-tata were stimulated for 8 h with anti-IgM (rdgM), PMA, or ionomycin, lysed, and luciferase activity was determined. B–D. DT40 cells were co-transfected with the 5xjun2-tata luciferase reporter and RasN17 (B), RalN28 (C), RalBPΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (rdgM), lysed, and luciferase activity was determined. A–D. Normalized reporter activity is shown as the mean ± S.D. 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 < 0.005). The results are representative of at least three independent experiments.
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 RalBP(GAP) (Fig. 6D), did not affect BCR-induced NF-κB activity. 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 control or a dominant-negative mutant RasN17 (B), RalN28 (C), or RalBP(GAP) (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 Ral. 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 control or a dominant-negative mutant RasN17 (B), RalN28 (C), or RalBP(GAP) (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, PLCγ2, the IP3-mediated Ca2+ 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 Ca2+ 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 RalN28 (by ~25%; Fig. 7C) or RalBP1GAP (by ~40%; Fig. 7D), indicating that Ral mediates Ras-controlled activation of NFAT in response to BCR stimulation.

**Discussion**

**BCR-controlled activation of Ral is mediated by Lyn/Syk, Btk, PLCγ2, IP3-mediated Ca2+ release, and Ras**

In this study, we have shown that BCR stimulation of primary B cells and B cell lines results in activation of Ral. BCR-controlled activation of Ral 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 Ral, because Ral activation was reduced in DT40 cells deficient in Btk (Fig. 2A). Btk mediates BCR-controlled activation of PLCγ2 (45), which leads to the generation of DAG and IP3. The latter binds IP3Rs and induces the release of Ca2+ from intracellular stores. Like Btk, PLCγ2 was found to be involved in activation of Ral, and because Ral activation was reduced in cells deficient for IP3Rs, this activation involves the PLCγ2-controlled increase of intracellular Ca2+ (Fig. 2, C and D). Furthermore, stimulation of DT40 B cells with PMA, which mimics the actions of DAG, did not result in Ral activation (Fig. 2E), indicating that the BCR-controlled production of DAG alone is not sufficient for activation of Ral. Notably, some activation of Ral still remains upon BCR stimulation of B cells deficient in Btk, PLCγ2, 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 Ral upon BCR stimulation (Fig. 3B), thereby demonstrating that Ras mediates activation of Ral by the BCR. The activation of Ras is impaired in PLCγ2-deficient...
cells, but not in IP<sub>P,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 RapGRP3 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 PLC<sub>γ2</sub>-deficient cells most likely involves Son-of-Sevenless (SOS) activity (66). Interestingly, the activation of Ras is impaired in PLC<sub>γ2</sub>-as well as IP<sub>P,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 RalBP<sub>α</sub> in alternative pathways, resulting in an increase in 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>α</sub> GAP. 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 possible inhibition of RalGEF-dependent but Ral-independent processes, we also made use of the RafBP<sub>α</sub> ΔGAP protein, which sequesters activated Ral and inhibits binding of Ral to its endogenous effector proteins (33).

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>α</sub> GAP 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>Rs (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 Ral activity is responsible for the BCR-induced expression of c-fos.

A major regulator of c-fos expression is the serum response factor, a downstream target of the Ras-ERK pathway. BCR activation controls serum response factor activity through Lyn, Syk, Btk, PLC<sub>γ2</sub>, 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 PLC<sub>γ2</sub>, 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 bind control elements of the NFAT promoter (77, 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 neoplasm 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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