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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 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 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) 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). Ral 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 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, Ral is able to activate NF-kB, 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 (PLC-γ2), the inositol-1,4,5-trisphosphate receptor (IP3/R), mediator of 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 Bioslabs); HRP-conjugated rabbit anti-mouse and HRP-conjugated goat anti-rabbit (both DakoCytomation).

Plasmids

pA-puroII-RasN17 (41) was provided by Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan). The 6xNFkB-RE- and 4xNFAT-RE-luciferase reporter constructs were obtained from Stratagene. pRK5-RalBP-GAP, pMT2-RAI-N17, pMT2-HA-RlI-CAAX, and the c-fos-luciferase, tata-luciferase, and 5xjun2-tata-luciferase reporter were described previously (30, 33, 34). 6xNFkB-RE-luciferase reporter and pMT2-HA-RFI-CAAX were provided by Dr. K. Reedquist (Academic Medical Center (AMC), Amsterdam, The Netherlands). pRK5-RalBP-GAP was provided by Dr. F. Zwartkruis (Utrecht Medical Center, Utrecht, The Netherlands). 5xjun2-tata-luciferase (34), tata-luciferase control (TATA), and c-fos-luciferase (42) reporters were provided by Dr. H. van Dam (Leiden University Medical Centre, Leiden, The Netherlands). 4xNFAT-RE-luciferase reporter was provided by Dr. P. Schirer (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 Medical Ethical Committee. Mouse splenic B cells were obtained 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 IP3R, 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)’, or 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, 20% glycerol, and 2× EDTA-free protease inhibitor 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 (100 μl of 20% solution per sample) were precoupled with GST-RalBP-Ral-binding domain (RBD) or GST-Ral-Ras-binding domain fusion protein by an equal volume mixing for 30 min at 4°C with bacterial cell lysates from Escherichia coli strain AD202 transformed with pGEX4T3-RalBP-RBD (43) or pE.coli strain BL21 transformed with pGEX2T-Raf-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-puroII-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 2 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 4xNFAT-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 × 105 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.

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
observed after BCR stimulation, demonstrating Btk is required for full Ral activation by the BCR (Fig. 2B).

PLCy2 is a substrate for Btk that mediates various BCR-controlled cellular responses (45). For example, BCR-controlled ERK activation is partly dependent on PLCy2 (41) and Ras activation in response to BCR triggering is severely reduced in DT40 cells deficient in PLCy2 (46) (see also Fig. 3A). To investigate whether PLCy2 is also involved in BCR-controlled Ral activation, PLCy2-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, PLCy2 activation leads to production of IP3, which results in IP3-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), \(\beta\)-arrestins (48), or Ras (14, 15). Similar to Ral, 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 Ral, 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 transcriptional activation 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 RalBP1, a Raf 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 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 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

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**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 (\(\alpha\)-IgM) Abs or PMA and ionomycin (P/I), lysed, and the amounts of Ras-GTP in the lysates was determined by pull-down assay using GST-Raf-RBD fusion protein (PD). As a control, total lysate (TL) proteins were immunoblotted and probed using anti-Ral Abs. B, WT and stably RasN17-transfected DT40 cells were stimulated for the indicated period of time with anti-IgM (\(\alpha\)-IgM) Abs, lysed, and the amounts of Ras-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.
transcriptional activity in DT40 WT cells, but not in cells deficient in Btk, PLCγ2, IP3Rs, Ras, and Ral. A. WT, Btk−, PLCγ2−, and IP3R− deficient DT40 cells transiently transfected with a c-fos-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 c-fos-luciferase reporter and RasN17 (B), RalN28 (C), RalBPGAP (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 < 0.01). The results are representative of at least three independent experiments.

FIGURE 5. BCR activation induces JUN/ATF2 activity through Btk, PLCγ2, IP3Rs, Ras, and Ral. A. WT, Btk−, PLCγ2−, and IP3R− deficient DT40 cells transiently transfected with the JUN-dependent luciferase reporter 5xjun2-tata 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 the 5xjun2-tata luciferase reporter and RasN17 (B), RalN28 (C), RalBPGAP (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 ± 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 activity by the BCR, DT40 cells were transiently transfected with a 6xNFκB-RE-luciferase reporter construct and stimulated for 8 h with anti-IgM (αIgM), PMA, or ionomycin, lyzed, and luciferase activity was determined. B–D, DT40 cells were cotransfected with a 6xNFκB-RE-luciferase reporter and RasN17 (B), RalN28 (C), RalBP3ΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (αIgM), lyzed, 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.

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 RalBP3ΔGAP (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, lyzed, and luciferase activity was determined. B–D, DT40 cells were cotransfected with a 6xNFκB-RE-luciferase reporter and RasN17 (B), RalN28 (C), RalBP3ΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (αIgM), PMA, or ionomycin, lyzed, 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, lyzed, and luciferase activity was determined. B–D, DT40 cells were cotransfected with a 4xNFAT-RE-luciferase reporter and RasN17 (B), RalN28 (C), RalBP3ΔGAP (D), or the corresponding control plasmid, and stimulated for 8 h with anti-IgM (αIgM), lyzed, 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\(_{\gamma2}\), and IP\(_3\)-mediated Ca\(^{2+}\) release. In addition, activation of Ras is required for Raf activation. In turn, Raf was found to be involved in the regulation of BCR-induced gene transcription by controlling AP-1 and NFAT activity. The proteins studied by using gene-deficient DT40 cells or inhibition by dominant-negative mutant expression are in gray. The connections described in this study are represented as bold arrows.

**FIGURE 8.** A schematic representation of the signaling pathway leading to Raf activation upon stimulation of the BCR is shown. The cytoplasmic kinases Lyn and Syk are required for activation of Ras, which is also mediated by Btk, PLC\(_{\gamma2}\), and IP\(_3\)-controlled Ca\(^{2+}\) release. In addition, activation of Ras is required for Raf activation. In turn, Raf was found to be involved in the regulation of BCR-induced gene transcription by controlling AP-1 and NFAT activity. The proteins studied by using gene-deficient DT40 cells or inhibition by dominant-negative mutant expression are in gray. The connections described in this study are represented as bold arrows.

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**Discussion**

**BCR-controlled activation of Raf is mediated by Lyn/Syk, Btk, PLC\(_{\gamma2}\), IP\(_3\)-mediated Ca\(^{2+}\) release, and Ras**

In this study, we have shown that BCR stimulation of primary B cells and B cell lines results in activation of Raf. BCR-controlled activation of Raf 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 \(\text{Xid}\) 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 Raf, because Raf 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 IP\(_3\). The latter binds IP\(_3\)Rs and induces the release of Ca\(^{2+}\) from intracellular stores. Like Btk, PLC\(_{\gamma2}\) was found to be involved in activation of Raf, and because Raf activation was reduced in cells deficient for IP\(_3\)Rs, this activation involves the PLC\(_{\gamma2}\)-controlled increase of intracellular Ca\(^{2+}\) (Fig. 2, C and D). Furthermore, stimulation of DT40 B cells with PMA, which mimics the actions of DAG, did not result in Raf activation (Fig. 2E), indicating that the BCR-controlled production of DAG alone is not sufficient for activation of Raf. Notably, some activation of Raf still remains upon BCR stimulation of B cells deficient in Btk, PLC\(_{\gamma2}\), and IP\(_3\)Rs, 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 Raf upon BCR stimulation (Fig. 3B), thereby demonstrating that Raf mediates activation of Raf by the BCR. The activation of Raf is impaired in PLC\(_{\gamma2}\)-deficient
cells, but not in IP₃R-deficient cells (Fig. 3A), indicating the involvement of DAG production rather than Ca²⁺ release in Ras activation. Indeed, the generation of DAG upon BCR stimulation can recruit the Ras exchange factor RasGRF3 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 IP₃R-deficient cells (Fig. 2, C and D). Besides the critical role for Ras in Ras activation, this also points toward an important role for a Ca²⁺/calmodulin-dependent signaling molecule (Figs. 8). A likely candidate is calmodulin, which has been shown to interact with Ras in a Ca²⁺-regulated manner, resulting in an increase of active Ras (47, 67).

**Ral mediates BCR-controlled gene transcription**

To study the possible role of Ras in BCR-controlled gene transcription, we made use of the dominant-negative RasN28 mutant and the mutant Ras effector RalBPAGAP. The RasN28 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 RasN28 as a consequence of steric hindrance or conformational changes induced by the binding of RasN28 to RalGEFs. Therefore, to exclude possible inhibition of RalGEF-dependent but Ras-independent processes, we also made use of the RalBPAGAP protein, which sequesters activated Ras and inhibits binding of Ras to its endogenous effector proteins (33).

One gene whose expression and function has previously been shown to be regulated by Ras is the c-fos proto-oncogene (30–32), which also acts downstream of the BCR (50). By expressing the dominant-negative RasN28 mutant or the mutant Ras effector RalBPAGAP we have demonstrated that Ras is involved in BCR-controlled transactivation of the c-fos promoter (Fig. 4, A and D). Although c-fos-luciferase reporter activity was not affected in cells deficient in IP₃R (Fig. 4A), it was impaired in cells transfected with a dominant-negative Ras (Fig. 4B), suggesting that Ras rather than Ca²⁺-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, Ca²⁺, 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 Ras mediates BCR-controlled activation of ATF2-dependent gene expression (Fig. 5, C and D), Ral may mediate expression of FOS through ATF2. Indeed, Ouwen 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 Ras is involved in activation of the JUN/ATF2-dependent luciferase reporter (Fig. 5), this suggests that Ras is involved in BCR-controlled regulation of JNK activity and JUN-mediated gene transcription. Taken together, Ras 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 Ras 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 B cell development 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 Ras. Indeed, we have demonstrated that Ras 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), Igκ (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 Ras has been implicated in activation of NFAT proteins, Ras 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 lymphocytic 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 neoplasma of terminally differentiated B cells which do not express BCR-expressing lymphomas. Indeed, whereas oncogenic Ras mutations have been detected in 40–50% of patients with multiple myeloma (82, 83), a neoplasma of terminally differentiated B cells which do not express

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fos
and activator protein-1.


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