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

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Bruton’s Tyrosine Kinase and Phospholipase Cγ2 Mediate Chemokine-Controlled B Cell Migration and Homing

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SUMMARY
Control of integrin-mediated adhesion and migration by chemokines plays a critical role in B cell development, differentiation, and function; however, the underlying signaling mechanisms are poorly defined. Here we show that the chemokine SDF-1 induced activation of Bruton’s tyrosine kinase (Btk) and that integrin-mediated adhesion and migration in response to SDF-1 or CXCL13, as well as in vivo homing to lymphoid organs, was impaired in Btk-deficient (pre-)B cells. Furthermore, SDF-1 induced tyrosine phosphorylation of Phospholipase Cγ2 (PLCγ2), which, unlike activation of the migration regulatory GTPases Rac or Rap1, was mediated by Btk. PLCγ2-deficient B cells also exhibited impaired SDF-1-controlled migration. These results reveal that Btk and PLCγ2 mediate chemokine-controlled migration, thereby providing insights into the control of B cell homeostasis, trafficking, and function, as well as into the pathogenesis of the immunodeficiency disease X-linked agammaglobulinemia (XLA).

INTRODUCTION
Integrin-mediated cell adhesion and migration play a critical role in a wide variety of processes underlying proper organization and function of the immune system, including B cell development and differentiation. During early B cell development, the consecutive generation of pro-, pre-, and immature B cells requires their retention in defined microenvironments in the bone marrow (BM), which is controlled by interactions of integrin α4β1 with fibronectin (FN) in the extracellular matrix (ECM) and with vascular cell adhesion molecule-1 (VCAM-1)-expressing BM stromal cells (Arroyo et al., 1999; Leuker et al., 2001; Rose et al., 2002; Tokoyoda et al., 2004). In mature B cells, integrins α4β1 and leukocyte function-associated antigen-1 (LFA-1) mediate high endothelial venule (HEV) attachment and transendothelial migration required for recirculation and homing, and they control cell compartmentalization in peripheral lymphoid tissue (Cyster, 2003, 2005; Koni et al., 2001; Miyasaka and Tanaka, 2004; Rose et al., 2002). Integrins α4β1 and LFA-1 also play a key role in the T cell-dependent humoral immune response, being involved in migration of naive B cells into B cell follicles and in the interaction of germinal center (GC) B cells with antigen-presenting follicular dendritic cells (FDCs) during antigen-specific B cell differentiation (Brakebusch et al., 2002; Koopman et al., 1991, 1994; Leuker et al., 2001; Rose et al., 2002; Spaargaren et al., 2003).

Chemokines play a prominent role in controlling integrin-mediated adhesion and migration (Kinashi, 2005; Laudanna et al., 2002). As such, the so-called homeostatic chemokines SDF-1 (CXCL12) and CXCL13 (BLC or BCA-1) and their respective G protein-coupled receptors CXCR4 and CXCR5 play a major role in B cell homeostasis, trafficking, and function (Campbell et al., 2003; Cyster, 2003, 2005; Miyasaka and Tanaka, 2004). CXCR4 is expressed by all B cell subsets (Bowman et al., 2000), and SDF-1 is highly expressed by stromal cells in the BM and GCs, by reticulum cells aligning the GCs, and in the splenic red pulp and lymph node medullary cords, and it is present on HEV in lymph nodes and Peyer’s patches (Allen et al., 2004; Cyster, 2003, 2005; Okada et al., 2002). SDF-1-CXCR4 signaling plays a critical role in a variety of processes underlying proper B cell development and function, including development and retention of precursor B cells in the BM, homing of mature B cells to secondary lymphoid organs, trafficking and homing of plasma cells to BM, GC organization, and T-independent humoral immune responses (Allen et al., 2004; Cyster, 2003, 2005; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004; Okada et al., 2002; Tokoyoda et al., 2004). CXCR5 is mainly expressed by mature B cells (Bowman et al., 2002).
CXCL13 is produced in follicles and is present on HEV of lymph nodes and Peyer’s patches (Miyasaka and Tanaka, 2004). CXCL13-CXCR5 signaling is required for migration of naive B cells into follicles and for GC organization (Allen et al., 2004; Ansel et al., 2000; Campbell et al., 2003; Cyster, 2005; Forster et al., 1996; Okada et al., 2002).

Despite the important role of chemokine-controlled integrin-mediated migration in B cell development and function as well as in the pathogenesis of B cell malignancies and chronic inflammatory or autoimmune diseases, the underlying signal transduction mechanisms are as yet poorly defined (Kinashi, 2005; Laudanna et al., 2002). Interestingly, many of the B cell defects in mice deficient in SDF-1–CXCR4, CXCL13–CXCR5, VCAM-1, or α4 integrin, such as impaired development and retention of B cell precursors in the BM and impaired B cell differentiation and immune responses (Allen et al., 2004; Ansel et al., 2000; Arroyo et al., 1999; Forster et al., 1996; Leuker et al., 2001; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004; Okada et al., 2002), are similar to the defects observed in the immunodeficiency diseases X-linked immunodeficiency (Xid) in mice and XLA in men, caused by loss-of-function germline mutations in the cytoplasmic tyrosine kinase Btk (Cariappa et al., 1999; Conley et al., 2005; Ellmeier et al., 2000; Hendriks et al., 1996; Khan et al., 1995; Middendorp et al., 2002; Nomura et al., 2000). Combining this notion with our recent finding that Btk is required for the control of integrin-mediated adhesion by the B cell antigen receptor (BCR) (Spaargaren et al., 2003), we hypothesized that Btk may be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration. Here, we demonstrate that SDF-1– or CXCL13-controlled integrin-mediated adhesion and migration, as well as in vivo homing, of pre-B and B cells was indeed mediated by Btk. Furthermore, Btk mediated SDF-1-induced phosphorylation of PLCγ2, and PLCγ2 mediated SDF-1-controlled migration as well.
This function for Btk implies that impaired chemokine-controlled migration may contribute to the developmental and functional B cell defects observed in XLA and Xid.

RESULTS

SDF-1 and CXCL13 Induce Integrin-Mediated Migration of B Cells

To study the control of integrin-mediated B cell migration by the chemokines SDF-1 and CXCL13, murine BM-derived pre-B cells, murine splenic B cells, and human tonsillar B cells were assayed for migration toward these chemokines in a transwell system of which the membrane was either uncoated or coated with VCAM-1 or FN. Although SDF-1 strongly induced the migration of these primary B cells on uncoated membranes, migration toward SDF-1 was more pronounced (~3-fold increase) on membranes coated with VCAM-1 (Figures 1A–1C). Similar results were obtained for migration on FN (not shown) and for migration of murine splenic B cells and tonsillar B cells toward CXCL13 (Figures 1B and 1C).

VCAM-1 and FN (not shown) also enhanced migration toward SDF-1 of the human GC B cell-like cell line Namalwa (Figure 1D) and the chicken DT40 B cells (Figure 1E) by approximately 3-fold. As shown in Figure 1F, the integrin $\alpha_4\beta_1$-blocking antibody HP2/1 and the $\alpha_4\beta_1$-activating antibody TS2/16 completely abolished VCAM-1-mediated migration of Namalwa cells toward SDF-1, whereas no effect was observed with either an isotype control IgG1 or the integrin $\alpha_4\beta_1$-blocking antibody Act-1. Furthermore, antibody CSAT, directed against the chicken integrin $\beta_1$ subunit, strongly suppressed SDF-1-induced
migration of DT40 cells on VCAM-1 (Figure 1G). Taken together, our data demonstrate that SDF-1 and CXCL13 control integrin (α4β1)-mediated migration of primary B cells and B cell lines.

**Btk Mediates Chemokine-Controlled Migration**

Because many of the defects observed in mice deficient in SDF-1-CXCR4, CXCL13-CXCR5, VCAM-1, or α4 integrin are also observed in Xid or Btk-deficient mice and XLA patients (as elaborated in the Discussion), and because we have recently established that Btk is required for the control of integrin-mediated adhesion by the BCR (Spaargaren et al., 2003), we hypothesized that Btk might be involved in the signaling mechanism underlying chemokine-controlled integrin-mediated migration.

In support of a possible role for Btk in chemokine-controlled migration, we observed SDF-1-induced tyrosine phosphorylation of Btk in DT40 cells, Namalwa cells, and human tonsillar B cells (Figure 2A). Phosphorylation of Btk could be detected with either a general phosphotyrosine antibody or a phospho-specific antibody for the autophosphorylation site Y223, reflecting Btk activation. Typically, SDF-1 induced Btk phosphorylation in a fast and transient fashion, with optimal phosphorylation being observed between 30 s and 2 min. A similar degree of Btk phosphorylation was observed after suboptimal duration of BCR stimulation (i.e., 2 min) with anti-IgM (Figure 2A); however, optimal 5 min BCR stimulation resulted in stronger Btk phosphorylation (not shown).

To directly examine a possible role for Btk in SDF-1-controlled integrin-mediated migration, we used pre-B cells derived from Btk-deficient mice. Interestingly, as shown in Figure 2B, Btk-deficient pre-B cells exhibited ~55% reduced SDF-1-induced migration on VCAM-1 in comparison to WT pre-B cells. Notably, surface expression of CXCR4, integrin α4 and β1 was similar on WT and Btk-deficient pre-B cells (see Figure S1 in the Supplemental Data available online). In DT40 cells, Btk deficiency also resulted in a reduction (by ~45%) of SDF-1-controlled migration (Figure 2C). This defect was not due to clonal variation because it could be completely restored by stable transfection of a Btk expression construct (Figure 2C). Furthermore, as shown in Figure 2D, whereas SDF-1-induced activation of the MAP kinase ERK2 (MAPK1) was slightly reduced, activation of PKB (Akt) was not affected in the Btk-deficient cells, demonstrating that the impaired migration is not due to a general signaling defect. To examine the role of Btk in the control of B cell migration by CXCL13, we used splenic B cells, because pre-B cells and DT40 cells do not respond to CXCL13 (not shown). As shown in Figure 2E, Btk-deficient B cells also exhibited impaired migration on VCAM-1 toward CXCL13 (by ~30%). Notably, surface expression of CXCR5 and integrin β1 was similar on WT and Btk-deficient splenic B cells (Figure S2).

In conclusion, these data identify Btk as a component of the SDF-1-CXCR4 signaling cascade and reveal a regulatory function for Btk in chemokine-controlled integrin-mediated migration of (pre-)B cells.
Btk-deficient mice, we used an IL-7-driven BM culture system to obtain better developmentally matched WT and Btk-deficient pre-B and immature B cell populations. 3 hr after adoptive transfer, lymphoid organs were collected to determine the homing ratio of the Btk-deficient B cells compared to WT cells (Figure 4A). Interestingly, the accumulation of the Btk-deficient pre-B cells was reduced in axillary (by ~45%), inguinal (~55%), and mesenteric (~60%) lymph nodes, the spleen (~25%), and in BM (~25%) (Figure 4B). Btk-deficient IgM+ immature B cells also exhibited impaired homing to the peripheral (by ~40%-~45%) and mesenteric (~70%) lymph nodes, but not to the spleen or BM (Figure 4C). Consistent with a homing defect, and opposing a possible survival disadvantage, elevated numbers of Btk-deficient B cells were recovered from the peripheral blood (Figure 4). These results clearly demonstrate that Btk-deficient pre-B and immature B cells suffer from an intrinsic defect in the in vivo homing to (secondary) lymphoid organs, characteristic of impaired chemokine-controlled migration.

The Role of Lyn and Syk Tyrosine Kinases and PI3K in SDF-1-Controlled Migration
Recent studies have revealed an important role for cytoplasmic tyrosine kinases in signaling by G protein-coupled receptors, and the cytoplasmic tyrosine kinases Lyn and Syk play a prominent role in BCR-controlled activation of Btk (Kurosaki, 2002). Consistent with a putative similar role for these kinases in SDF-1-induced activation of Btk, SDF-1-controlled migration on VCAM-1 was reduced by ~50% in DT40 cells deficient in both Lyn and Syk (Figure S3A and Supplemental Results).

Previous studies have also implicated PI3K in chemokine-controlled migration (Kinashi, 2005; Laudanna et al., 2002) and in activation of Btk (Kurosaki, 2002). However, in comparison to the reduced migration in Btk-deficient DT40 cells (~45%) (Figure 2C), pretreatment of DT40 or Namalwa cells with the unrelated PI3K inhibitors Wortmannin (WM) and LY294002 (LY) caused only a minor reduction of SDF-1-induced migration (~20%) (Figure S4B and Supplemental Results). Yet, the residual migration of
the Btk-deficient cells toward SDF-1 could be further reduced by ~60% upon PI3K inhibition (Figure S4B). Taken together, these results suggest that PI3K and Btk mediate SDF-1-controlled migration in a parallel fashion, independent of each other.

**Btk Mediates SDF-1-Induced Phosphorylation of PLCγ2 but Not Activation of Rap1 or Rac**

Next, we wished to explore which signaling molecules may control chemokine-controlled migration downstream of Btk. The small GTPases Rap1 and Rac have both been implicated in SDF-1-controlled migration of B lymphocytes (Kinashi, 2005; McLeod et al., 2002). Indeed, GTPase pull-down assays revealed that SDF-1 stimulation of DT40 cells resulted in enhanced amounts of GTP-bound Rap1 and Rac; however, similar Rap1 and Rac activation was observed upon SDF-1 stimulation of Btk-deficient DT40 cells (Figure 5A). Thus, SDF-1-induced activation of neither Rap1 nor Rac is mediated by Btk.

Another candidate is PLCγ2, which has been identified as a direct substrate for Btk (Kurosaki, 2002). Furthermore, we have recently established an important role for PLCγ2 in BCR-controlled integrin-mediated adhesion (Spaargaren et al., 2003). Interestingly, SDF-1 stimulation resulted in enhanced tyrosine phosphorylation of PLCγ2 in Namalwa cells, human tonsillar B cells, and DT40 cells (Figures 5B and 5C). SDF-1-induced phosphorylation of PLCγ2 could be detected with a general phosphotyrosine antibody as well as by phospho-specific PLCγ2 antibodies directed against Y1217 and the Btk substrate site Y759. Importantly, SDF-1-induced tyrosine phosphorylation of PLCγ2 was severely reduced in the Btk-deficient DT40 cells and could be completely restored by stable transfection of a Btk expression construct (Figure 5C). Most likely because of higher Btk expression, PLCγ2 phosphorylation was even more pronounced in these reconstituted cells. Furthermore, SDF-1-induced phosphorylation of PKB (analyzed in the same cell lysates) was not affected by the absence of Btk (Figure 5C), thereby emphasizing the specificity of the defect in PLCγ2 phosphorylation. Thus, these data demonstrate that Btk mediates SDF-1-induced phosphorylation of PLCγ2.

**PLCγ2 Mediates SDF-1-Controlled Migration**

To examine a possible role for PLCγ2 in SDF-1-controlled migration, we used PLCγ2-deficient DT40 cells. As shown in Figure 6A, PLCγ2-deficient DT40 cells showed a reduction of SDF-1-controlled migration on VCAM-1 by ~55%. This defect could be completely restored by stable transfection of a PLCγ2 expression construct (Figure 6A), demonstrating that the impaired migration is not due to clonal variation. Similar to Btk-deficient DT40 cells (Figure 5C), SDF-1-induced phosphorylation of PKB was not affected, but phosphorylation of ERK2 was reduced in PLCγ2-deficient DT40 cells (Figure 6B). Interestingly, treatment of Namalwa or DT40 cells with the PLC inhibitor U73122 almost completely abolished SDF-1-induced migration on VCAM-1 (Figure 6C). These data indicate that PLCγ2, together with other isoforms of PLC, plays an important role in SDF-1-CXCR4 signaling and SDF-1-controlled B cell migration.

**DISCUSSION**

Our results reveal that Btk and PLCγ2 mediate chemokine-controlled integrin-mediated migration. Besides...
providing several answers and new insights, this study also raises some new questions and challenges for future studies.

How do chemokines control Btk activity? In BCR signaling, the PI3K product PIP3 is implicated in membrane recruitment of Btk, enabling activation of Btk by Lyn, whereas Syk, through phosphorylation of the adaptor BLNK (SLP-65 or BASH), facilitates activation of PLCγ2 by Btk (Kurosaki, 2002). Consistent with a putative role for these tyrosine kinases in activation of Btk and/or PLCγ2 by SDF-1, SDF-1-controlled migration was impaired in B cells deficient in Lyn and Syk. In contrast, our observations suggest that PI3K does not mediate activation of Btk by SDF-1. Yet, SDF-1-induced membrane recruitment of a Btk-GFP fusion protein in Hela cells was reported to be sensitive to PI3K inhibition (Nore et al., 2000). However, supporting our results, and opposing the general perception that activation of Btk is strictly dependent upon its PI3K-mediated membrane recruitment, BCR-controlled phosphorylation and activation of Btk (and PLCγ2) is not affected in B cells deficient in the regulatory PI3K subunit p85α or the catalytic subunit p110γ, nor in primary B cells treated with LY or WM (Jou et al., 2002; Suzuki et al., 2003). Likewise, in accordance with the minor effect of PI3K inhibition on SDF-1-induced B cell migration, several recent studies have challenged the general concept that PI3K activation is indispensable for chemokine-controlled cell migration (Nombela-Arrieta et al., 2004; Smit et al., 2003; Ward, 2004).

Nevertheless, the residual migration of Btk-deficient cells was largely PI3K dependent. Most likely, this involves the catalytic p110δ and adaptor p85α subunits (Figure 7), which, in contrast to the catalytic subunit p110γ, have been implicated in chemokine-controlled B cell migration (Nombela-Arrieta et al., 2004; Reif et al., 2004). Recently, B cells were shown to require the scaffolding protein DOCK2 for efficient SDF-1- and CXCL13-controlled migration and integrin-mediated adhesion to ICAM-1 and VCAM-1, respectively (Kinashi, 2005; Nombela-Arrieta et al., 2004). DOCK2, like Btk, mediates chemokine-controlled migration in a largely PI3K-independent fashion (Nombela-Arrieta et al., 2004), so it is tempting to speculate that DOCK2 may be involved in chemokine-induced Btk or PLCγ2 activation (Figure 7). Alternatively, by analogy to

Figure 6. PLCγ2 Mediates SDF-1-Controlled Migration
(A) Wild-type (WT), PLCγ2-deficient (PLCγ2−/−), or PLCγ2-deficient DT40 cells reconstituted with PLCγ2 (PLCγ2+) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1, as indicated (n = 6). The bars represent the means ± SD of six independent experiments, each performed in triplicate.

(B) Activation of PKB and ERK in wild-type (WT) or PLCγ2 (PLCγ2−/−)-deficient DT40 cells after stimulation with SDF-1 for the indicated period of time (min). The blots are probed as in Figure 2D.

(C) Namalwa cells (left) or DT40 cells (right) were pretreated with 2.5 μM U73122 (U) or left untreated for 30 min, and subsequently cells were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in transwells coated with 1 μg/ml VCAM-1 (n = 5). The bars represent the means ± SD of five independent experiments, each performed in triplicate.
Immunity

Btk and PLCγ2 Regulate B Cell Migration

How do Btk and PLCγ2 control chemokine-induced migration? Our results strongly indicate that Btk mediates chemokine-controlled migration through PLCγ2, which is highly expressed in all B cell subsets (Figure 7). To our knowledge, the only previous evidence for a possible role for PLCγ1 in chemokine signaling is the observation that SDF-1 induces tyrosine phosphorylation of PLCγ1 (isotype undefined) in hematopoietic progenitor cells (Wang et al., 2000). In addition, since SDF-1-induced migration could be completely abolished with the general PLC inhibitor U-73122, other PLC isotypes, such as PLCγ1, which is highly expressed in pro- and pre-B cells, or PLCδ1, may be involved as well (Figure 7). Similar to our observations, CXCR3-mediated migration of T lymphocytes is relatively insensitive to PLCδ3 inhibition, but can be completely abolished by PLC inhibition (Smit et al., 2000). The observed critical role of PLC, including PLCγ2, in chemokine-controlled migration points toward an important downstream role for calcium- and/or DAG-dependent signaling molecules. Likely candidates are the classical and novel isoforms of PKC and the RasGRP (CalDAG-GEF) family of exchange factors, which act on different Ras family GTPTases, including Ras and Rap1 (Kinashi, 2005). Indeed, Rap1 was shown to be involved in SDF-1-induced migration of B lymphocytes (McLeod et al., 2002; Shimonaka et al., 2003). However, SDF-1-induced activation of Rap1 was not impaired in Btk- or PLCγ2-deficient B cells. Similarly, activation of Rap1 by SDF-1 is not impaired in Jurkat T cells deficient in PLCγ1, the major PLCγ isotype in T cells (Katagiri et al., 2004). The activation of Rac, another GTPTase implicated in chemokine-controlled migration, can occur through PI3K-dependent and PI3K-independent mechanisms (Kinashi, 2005; Ward, 2004). Noteworthy, DOCK2 also mediates activation of Rac (and Rap) (Nombela-Arrieta et al., 2004; Sanui et al., 2003). However, SDF-1-induced activation of Rac was not impaired in Btk- or PLCγ2-deficient B cells. Taken together, our data indicate that Btk, Rap1, and Rac act in parallel signaling pathways in chemokine-controlled B cell migration (Figure 7).

What about the role of other Tec family kinases in chemokine-controlled migration? Several members of the Tec family of tyrosine kinases, consisting of Tec, Btk, Itk, Rik, and Bmx, have previously been implicated in cytoskeletal reorganization, integrin-mediated adhesion, or migration (Berg et al., 2005). In B cells, we have previously shown that Btk mediates BCR-controlled α4β1-mediated adhesion, which involves cytoskeletal reorganization (Spaargaren et al., 2003). Similarly, in T cells, Itk mediates TCR-controlled actin polymerization and activation of β1 integrins (Berg et al., 2005). Moreover, two recent studies revealed a critical role for the Tec family kinases Itk and Rik in chemokine-controlled migration of T cells (Fischer et al., 2004; Takesono et al., 2004). Furthermore, in neutrophils, the chemotactic peptide fMLP induces the membrane recruitment and activation of Tec, Btk, and Bmx (Lachance et al., 2002), which, like the chemotactic response, can be suppressed by the pharmacological Tec family kinase inhibitor LFM-A13 (Gilbert et al., 2003). Recently, LFM-A13 was also reported to inhibit SDF-1-induced B cell migration and homing, and solely based upon the use of this inhibitor, the authors propose a role for Btk in these processes (Ortolano et al., 2006). We would like to emphasize, however, that LFM-A13 is not a specific Btk inhibitor: it is a potent inhibitor of the other Tec family kinases as well (Chau et al., 2005; Fernandes et al., 2005; Gilbert et al., 2003), and LFM-A13 is an equally efficient inhibitor of the nonrelated kinase JAK2 (van den Akker et al., 2004), which has also been implicated in SDF-1-induced lymphocyte migration (Soriano et al., 2003; Zhang et al., 2001). Yet, combining all of the above with our current findings, Tec family kinases appear to play an important role in chemokine-controlled adhesion and migration, at least in lymphocytes. Given the observed partial redundancy of Btk with Tec in murine B cells (Eilmeier et al., 2000), it would be interesting to determine whether Tec (or another Tec family member) is responsible for the residual migration observed in Btk-deficient (pre)J-B cells. Furthermore, by analogy to the role of Btk and PLCγ2 in chemokine-controlled B cell migration, it would be interesting to determine whether chemokine-controlled migration of T lymphocytes involves Itk- or Rik-mediated phosphorylation and activation of PLCγ1, the major PLCγ isotype in T cells.
What are the implications for XLA and Xid? Our study demonstrates a direct role for Btk in signaling by CXCR4 and in chemokine-controlled adhesion, migration, and homing. Interestingly, loss-of-function germline mutations in the BTK gene give rise to the B cell immuno-deficiency disease XLA in humans and Xid in mice. XLA patients show a severe reduction in mature B cell numbers (>99%) (Conley et al., 2005; Nomura et al., 2000), and in Xid- and the phenotypically identical Btk-deficient mice, mature B cell numbers are ~50% reduced (Khan et al., 1995). The earliest role for Btk occurs during B cell development at the progression from pre-B to immature B cells, which is severely impaired in XLA patients and to a lesser extent in Xid and Btk-deficient mice (Conley et al., 2005; Hendriks et al., 1996; Middendorp et al., 2002). In XLA, this involves a proliferation defect of the γH-chain-positive pre-B cells (Nomura et al., 2000), and in Xid mice this involves a defect in cellular maturation of pre-B cells (Middendorp et al., 2002). Furthermore, Xid mice have been reported to display a defect in the retention of immature B cells in the BM (Cariappa et al., 1999). Interestingly, −/− integrin-deficient pre-B cells also display a proliferation defect (Arroyo et al., 1999), and SDF-1 was originally identified as a pre-B cell growth-stimulating factor. Moreover, our results as well as previous studies (Bowman et al., 2000; Tokoyoda et al., 2004) show that SDF-1 controls α4β1-mediated adhesion and migration of pre-B and immature B cells, and mice deficient in SDF-1-CXCR4, VCAM-1, or −/− integrin display defects in development and retention of precursor (pro-, pre-, and immature) B cells in the BM (Arroyo et al., 1999; Leuker et al., 2001; Ma et al., 1999; Nagasawa et al., 1996; Nie et al., 2004). In the mature B cell population, Btk-deficient mice reveal a defect in follicular entry of long-lived recirculating follicular cells, in follicle and GC formation, in T-independent immune response, and a strongly reduced primary and variably affected secondary T-dependent immune response, whereas both responses are absent in Btk/Tec double-deficient mice (Ellmeier et al., 2000; Hendriks et al., 1996; Khan et al., 1995; Ridderdstad et al., 1996; Vinuesa et al., 2001). Similarly, mice deficient in either SDF-1 or CXCR4 and mice deficient in either CXCL13 or CXCR5 display defects in migration of naive B cells into follicles, GC B cell migration, and GC organization (Allen et al., 2004; Ansel et al., 2000; Forster et al., 1996; Okada et al., 2002). CXCR4-deficient mice show a loss of T-independent immune response (Nie et al., 2004), and mice lacking either VCAM-1 expression on FDCs or expression of integrin β1 in the hematopoietic system exhibit an impaired T-dependent immune response (Brakebusch et al., 2002; Koni et al., 2001; Leuker et al., 2001). Thus, based upon our current findings, impaired chemokine (SDF-1 or CXCL13)-controlled adhesion and migration, required for localization of pre-B and immature B cells in the appropriate BM niches and for emigration of immature B cells from the BM into the blood and to secondary lymphoid organs, may very well contribute to the defects in early B cell development in XLA and Xid and to the partial defects in localization, differentiation, and responses of mature B cells in Xid mice.

In conclusion, our results demonstrate that Btk and PLCγ2 mediate chemokine-controlled B cell adhesion and migration, which play an important role in B cell development and function as well as in the pathogenesis of B cell malignancies and chronic inflammatory or autoimmune diseases. Furthermore, our results indicate that impaired adhesion and migration, resulting from loss-of-function germline mutations of BTK, may contribute to the developmental and functional B cell defects observed in XLA patients and Xid mice.

EXPERIMENTAL PROCEDURES

See Supplemental Experimental Procedures online for complete methods.

Materials

The following reagents were used in this study: phosphorylation state-specific antibodies phospho-p44/42 MAP kinase (T202/Y204), phospho-Akt (S473), phospho-Btk (Y223), phospho-PLCγ2 (Y759), and phospho-PLCγ2 (Y1217) (Cell Signaling Technology); phosphotyrosine antibodies PY20 (BD Biosciences) and 4G10 (Upstate Biotechnology); rabbit polyclonal antibodies against ERK2 (C-14), Akt1/2 (H-138), Btk (E20), PLCγ2 (I20 and H160) (Santa Cruz Biotechnology), and CXCR4 (AB 1846) (Chemicon); mouse monoclonal antibodies against T7 (Novagen), Btk (G149-11), Rap1, and Rac1 (BD Biosciences); mouse monoclonal IgG1 antibodies PH2/2 against integrin subunit α4 (Immunotech), Act-1 against integrin subunit β1 (kindly provided by A. Lazaro-vits), TS2/16 against integrin subunit β1 (kindly provided by F. Sanchez-Madrid), CSAT IgG2b against the chicken integrin subunit β1 (DSHB, University of Iowa); rat and hamster monoclonal antibodies PS2/2-biotin and OXM718-FITC against mouse integrin α4 and β1, respectively (Chemicon), and 2G8-biotin against CXCR5 (BD Biosciences); anti-CD45RB/BB20 microbeads (Miltenyi Biotec); mouse anti-human IgM (MH15) (Sanquin, Amsterdam, the Netherlands), goat anti-chicken IgM (Bethyl Laboratories); anti-B220-FITC (Leuco Technologies), anti-IgM-biotin (BD Biosciences), Streptavidin-PE, Streptavidin-FITC, goat anti-mouse-PE (Southern Biotechnologies), anti-IgM-biotin (BD Biosciences), Streptavidin-PE, Streptavidin-FITC, goat anti-mouse-Biotin, goat anti-mouse-PE (Southern Biotechnologies), Swine anti-Rabbit-FITC (DAKO), and rabbit anti-goat-Biotin (Vector); pharmacological inhibitors PD-98059, LY-29402, Wortmannin, U-73122 (Biomol); recombinant human sVCAM-1, SDF-1α and BCA-1/CXCL13 (R&D Systems), human plasma FN, and BSA (fraction V) (Sigma); CMFDA and CMTMR (Molecular Probes).

Isolation of Tonsillar B Cells, Murine Pre-B Cells, and Splenic B Cells

Human tonsillar B cells, mouse splenic B cells from Btk-deficient mice and WT littermate controls, and mouse pre-B cells (IL-7-driven primary BM cultures of WT and Btk-deficient mice) were obtained essentially as described (Koopman et al., 1994; Middendorp et al., 2002; Spaargaren et al., 2003).

Cell Migration Assay

Migration assays were performed in triplicate with transwells (Costar) coated with 1μg/ml sVCAM-1 or 10μg/ml FN. The lower compartment contained 100 ng/ml SDF-1 or 500 ng/ml CXCL13, and the cells were applied to the upper compartment and allowed to migrate for 5 hr (DT40 and Namalva), 3.5 hr (tonsil B cells), 2.5 hr (splenic B cells), or 1 hr (pre-B cells) at 37°C (39.5°C for DT40), unless otherwise indicated. The amount of viable migrated cells was determined by FACs and expressed as a percentage of the input. Unless otherwise indicated, the migration of nonpretreated WT cells on VCAM-1-coated transwells in the presence of SDF-1 or CXCL13 was normalized to 100%, and the bars represent the means ± SD of at least three independent experiments, each assayed in triplicate.
Cell Adhesion Assays
The static cell adhesion to VCAM-1 was assayed essentially as described (Spaargaren et al., 2003), except that 150 ng/ml SDF-1 was coimmobilized with 1 µg/ml VCAM-1 on the plates, the plates were spun directly after applying the cells to the plate, and the cells were allowed to adhere for 2 min.

The under flow cell adhesion to HUVECs was assayed essentially as described (da Costa Martins et al., 2006). In brief, primary HUVECs were overlaid with 10 ng/ml SDF-1 prior to perfusion, DT40 cells were perfused at 0.8 dyn/cm² for 5 min, and 20 randomized images were recorded between 2 and 5 min were analyzed to determine the average amount of adhering cells per field.

In both assays, the adhesion of WT cells in the presence SDF-1 was normalized to 100%.

Homing Assay
Cells from IL-7-driven BM cultures of WT and Btk-deficient mice were labeled with either CMFDA or CMTMR, mixed 1:1, and injected intravenously in C57Bl/6 mice. Each WT/Btk-/- combination was analyzed by adoptive transfer of two recipient mice, which included a dye-swap. After 3 hr, lymphoid organs were collected and FACS analyzed to identify pre-B and immature B cells by their B220/IgM profile and to quantify dye-labeled cells. The homing ratio (Btk-deficient/WT cells) was corrected for the input ratio (which was normalized to 1).

Immunoprecipitation and Immunoblotting
For analysis of Btk and PLCγ2 tyrosine phosphorylation, cells were stimulated with 200 ng/ml SDF-1 or 10 µg/ml anti-IgM and immunoprecipitated with anti-Btk (C20), anti-IT, anti-P-Btk, anti-PLCγ2 (Q20), or anti-phosphotyrosine (PY20 or 4G10). Immunoprecipitates and total lysates were analyzed by SDS-PAGE and immunoblotting with anti-P-Btk, anti-Btk (G149-11), anti-P-PLCγ2, anti-PLCγ2 (H160), or anti-phosphotyrosine (4G10).

For analysis of ERK and PKB phosphorylation, cells were stimulated with 100 ng/ml SDF-1 and immunoblotted with anti-phospho-MAPK and anti-phospho-PKB, as described (Spaargaren et al., 2003).

Rac and Rap1 Activity Pull-Down Assays
Cells were stimulated with SDF-1 (100 ng/ml), and cell lysates were prepared and used immediately for GTPase pull-down assays with GST-Rac-GDS-RBD or GST-PAK-RBD fusion protein for Rap and Rac, respectively. Bound proteins were eluted with sample buffer, separated by 15% SDS-PAGE, and immunoblotted with anti-Rap1 or anti-Rac1.

Statistical Analysis
The unpaired two-tailed Student’s t test was used to determine the significance of differences between means. Unless otherwise indicated, all relevant comparisons (e.g., control versus inhibitors or WT versus gene-deficient cells) were significantly different (p < 0.05).

Supplemental Data
Supplemental Data include four figures, Supplemental Results, and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/26/1/93/ DC1/.

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Supplemental Results

SDF-1-Controlled Migration Is Impaired in Lyn- and Syk-Deficient B Cells

Recent studies have revealed an important role for cytoplasmic tyrosine kinases in signaling by G protein-coupled receptors. Moreover, Lyn, a Src-family tyrosine kinase, and Syk, a ZAP70-family tyrosine kinase, play a prominent role in (BCR-)signaling in B cells, including in activation of Btk (Kurosaki, 2002). To investigate a possible role for Lyn and Syk in the regulation of integrin-mediated migration by SDF, we made use of DT40 cells deficient in both Lyn and Syk. As shown in Fig. S3A, in Lyn/Syk double-deficient DT40 cells the SDF-1-controlled migration on VCAM-1 was reduced by ~50%. SDF-1-induced phosphorylation of PKB and ERK2 was not affected in the Lyn/Syk-deficient cells, demonstrating that the reduced migration is not due to a general signaling defect (Fig. S3B). Thus, these data demonstrate that Lyn and/or Syk are involved in SDF-1-mediated migration, which is in line with a putative role for these kinases in SDF-1-induced activation of Btk.

Btk and PI3K Mediate SDF-Controlled Migration Independent of Each Other

Previous studies have implicated PI3K in chemokine signaling and chemokine-controlled migration (Kinashi, 2005; Laudanna et al., 2002). Furthermore, the PI3K product PIP3 has been implicated in membrane recruitment and activation of Btk by binding the PH domain of Btk (Kurosaki, 2002). To investigate the role of PI3K in SDF-1-controlled integrin-mediated migration we used the unrelated PI3K inhibitors Wortmannin (WM) and LY294002 (LY). Pretreatment of Namalwa or DT40 cells with LY and WM, but not with the MEK inhibitor PD98059, completely abolished SDF-1-induced phosphorylation of PKB (Fig. S4A). Interestingly, however, inhibition of PI3K caused only a minor reduction of migration by approximately 20% (Fig. S4B). Notably, we used as much as 40 μM LY and 200 nM WM, and parallel analysis of SDF-1-induced PKB phosphorylation during the course of the migration experiment confirmed full inhibition of PI3K activity (data not shown). In comparison to the small effect of PI3K inhibition (~20%), the loss of Btk in DT40 cells resulted in a more severe reduction of migration (~45%) (Fig. 2C), indicating that Btk mediates B cell migration in a (largely) PI3K-independent manner. SDF-1-induced activation of PI3K is not Btk-dependent either, since the completely

Supplemental Data
Bruton’s Tyrosine Kinase and Phospholipase Cγ2 Mediate Chemokine-Controlled B Cell Migration and Homing

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PI3K-dependent phosphorylation of PKB is not affected in the Btk-deficient cells (Figs. 2D and S4A). Moreover, the residual migration of Btk-deficient cells could still be further reduced upon treatment with LY and WM by approximately 60% (Fig. S4B). Taken together, these results suggest that PI3K and Btk mediate SDF-1-controlled migration in a parallel fashion, independent of each other.
Figure S1. Similar Expression Levels of CXCR4, Integrin α4 and β1 on WT and Btk-Deficient Pre-B Cells

WT and Btk-deficient mice pre-B cells were FACS-analyzed for expression of CXCR4, integrin α4 and β1, using AB 1846, PS/2-biotin, and OXM718-FITC antibodies, respectively.
WT and Btk-deficient splenic B cells were FACS-analyzed for expression of CXCR5 and integrin β1, using 2G8-biotin and OXM718-FITC antibodies, respectively. A representative result is shown.

**Figure S2. Similar Expression Levels of CXCR5 and Integrin β1 on WT and Btk-Deficient Splenic B Cells**
Figure S3. SDF-1-Controlled Migration Is Impaired in Lyn and Syk-Deficient B Cells

(A) Wild type (WT) or Lyn/Syk double-deficient DT40 cells (Lyn−/Syk−) were allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=4).

(B) Activation of PKB and ERK in wild type (WT) or Lyn/Syk double-deficient DT40 cells (Lyn−/Syk−) after stimulation with anti-IgM for the indicated period of time (min). The immunoblots are probed as in Fig. 2D.
Figure S4. Btk and PI3K Mediate SDF-1-Controlled Migration Independent of Each Other

(A) Activation of PKB and ERK in Namalwa cells (left) or DT40 cells (right) which were pretreated with 20 μM LY294002 (LY), 100 nM Wortmannin (WM), 50 μM PD98059 (PD) or left untreated (-) for 30 min, and stimulated with SDF-1 or not (C) for 5 min, as indicated. The blots are probed as in Fig. 2D.

(B) Namalwa cells (left), WT DT40 cells (middle), or Btk-deficient DT40 cells (Btk−) (right) were pretreated with 40 μM LY294002 (LY), 200 nM Wortmannin (WM), or left untreated for 30 min, and allowed to migrate in the absence (C) or presence of 100 ng/ml SDF-1 (SDF-1) in Transwells coated with 1 μg/ml VCAM-1 (n=5). NS = not significant.
Supplemental Experimental Procedures

Cell Lines and Generation of Reconstituted DT40 Cells

The Burkitt’s lymphoma cell line Namalwa, and the chicken bursal lymphoma B cell line DT40 and DT40 cells deficient in both Lyn and Syk, Btk, or PLCγ2, obtained from Riken Cell Bank (Tsukuba Science City, Japan) with permission from Dr. T. Kurosaki, were cultured as previously described (Spaargaren et al., 2003). The reconstitution of the Btk or PLCγ2-deficient DT40 cell lines was performed by electroporation, using pApuro expression vectors containing the T7-tagged cDNAs for human Btk and rat PLCγ2 (kindly provided by Dr. T. Kurosaki), exactly as described (Spaargaren et al., 2003). All DT40 cells, i.e., WT, gene-deficient and reconstituted, showed similar expression of surface IgM and integrin α1, as determined by FACS analysis, using goat anti-chicken IgM (10 μg/ml) and rabbit anti-goat-Biotin/Streptavidin-PE, or CSAT (3 μg/ml) and goat anti-mouse-PE, respectively, analyzed with a FACSCalibur™ (Beckton Dickinson) using CELLQuest Pro software.

Isolation of Tonsillar B Cells, Murine Pre-B Cells, and Splenic B Cells

Human tonsillar B cells were isolated essentially as previously described (Koopman et al., 1994; Spaargaren et al., 2003). Typically, the obtained cell population contained >97% B cells as determined by FACS analysis. Isolated B cells were maintained in RPMI containing 10% FCS and were used immediately or after overnight storage at 4°C. Mouse splenic B cells were obtained from C57BL/6 mice, bred and maintained at the animal care facility of the Academic Medical Center. In addition, mouse splenic B cells were from Btk-deficient mice, i.e., Btk−/−LacZ mice crossed onto a C57BL/6 background for over 6 generations and Btk genotyped as described (Hendriks et al., 1996), and littermate controls, bred and maintained at the animal care facility of the Erasmus MC (Rotterdam, the Netherlands). 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. Expression levels of CXCR5 and integrin β1 were similar on WT and Btk-deficient splenic B cells as determined by FACS analysis using 2G8-biotin (1:50) and OXM718-FITC (1:20) antibodies, respectively. To obtain mouse pre-B cells, IL-7-driven primary BM cultures of WT and Btk-deficient mice were performed essentially as described (Dingjan et al., 2001; Spaargaren et al., 2003). The IL-7 driven BM cultures of the WT or Btk-deficient mice typically resulted in >98% B220+ cells and approximately 80% and 95% pre-B cells (B220+IgM−), respectively. Expression levels of CXCR4, integrin α4 and β1 were similar on WT and Btk-deficient mice pre-B cells as determined by FACS analysis, using AB 1846 (1:25), PS/2-biotin (1:80), and OXM718-FITC (1:20) antibodies, respectively.
Cell Migration Assay

Migration assays were performed in triplicate using 5 μm (DT40 and primary B cells) or 8 μm (Namalwa) pore size Transwells (Costar), coated overnight at 4°C with PBS containing 1 μg/ml sVCAM-1 or 10 μg/ml FN, and washed twice with PBS and blocked for 1 hr at 37°C with 0.5% BSA in RPMI 1640. The lower compartment was filled with 600 μl 0.5% BSA/RPMI containing 100 ng/ml SDF-1 or 500 ng/ml CXCL13, and 5 x 10^5 cells in 100 μl were applied to the upper compartment and allowed to migrate for 5 hrs (DT40 and Namalwa), 3.5 hrs (tonsillar B cells), 2.5 hours (splenic B cells), or 1 hr (pre-B cells) at 37°C (39.5°C for DT40), unless otherwise indicated. Where indicated, cells were pretreated with pharmacological inhibitors for 30 min at 37°C, or with anti-integrin antibodies Act-1 (3 μg/ml), TS2/16 (1:5) and HP2/1 (1 μg/ml) for 1 hour at 4°C, in RPMI with 1% BSA, and inhibitors and antibodies were also present in the Transwells during migration. The amount of viable migrated cells was determined by FACS and expressed as a percentage of the input, i.e., the number of cells applied directly into the lower compartment in parallel wells. Unless otherwise indicated, the migration of non-pretreated WT cells on VCAM-1-coated Transwells in the presence of SDF-1 or CXCL13 was normalized to 100%, and the bars represent the means ± SD of at least 3 independent experiments, each assayed in triplicate.

Cell Adhesion Assays

The static cell adhesion to VCAM-1 was assayed essentially as described previously (Spaargaren et al., 2003), with the following modifications: 96-well flat-bottom high binding plates (Costar) were used; 150 ng/ml SDF-1 was co-immobilized with 1 μg/ml VCAM-1 on the plates; the plates were spun for 30 sec at 400 rpm directly after applying the cells to the plate, and; the cells were allowed to adhere for 2 min and plates were washed twice to remove non-adhering cells. The adhesion of WT cells on SDF-1/VCAM-1-coated wells was normalized to 100%, and the bars represent the means ± SD of 4 independent experiments, each assayed in triplicate.

The under flow cell adhesion to HUVECs was assayed essentially as described previously (da Costa Martins et al., 2006). Briefly, primary human umbilical cord vein endothelial cells were isolated, cultured for 3 or 4 passages, and stimulated for 5 hrs with 10 ng/ml TNFα (Pepro Tech); The HUVECs were overlaid with 10 ng/ml SDF-1 for 5 min at 37°C prior to perfusion; DT40 cells (10^6/ml) were perfused at 0.8 dyn/cm^2 for 5 min; images were recorded for 5 sec from 2 to 5 minutes after the start of perfusion; 20 randomized fields were analyzed to determine the average amount of adhering cells per field. The adhesion of WT cells on SDF-1-overlaid HUVECs was normalized to 100%, and the bars represent the means ± SD of 3 independent experiments, each assayed in duplicate.
**Homing Assay**

WT and Btk-deficient pre-B and immature B cells were obtained from IL-7-driven BM cultures: Pre-B cell fractions were harvested after 4 days of culture in the presence of 100 U/ml IL-7, whereas immature B cell fractions were collected after one additional day of culture without IL-7. WT and Btk-deficient cells were labeled for 45 min at 37°C either with 0.25 μM Cell tracker Green (CMFDA) or 2 μM Cell tracker Orange (CMTMR), washed, and WT and Btk-deficient cells (15 x 10^6 cells each) were mixed and injected intravenously in age- and sex-matched C57BL/6 mice. After 3 hrs, blood, lymph nodes, spleen and BM were collected, and FACS-analyzed to identify pre-B and immature B cells by their B220/IgM profile and to quantify dye-labeled cells. Each combination of cells from a WT and a Btk-deficient mouse was analyzed by adoptive transfer of 2 recipient mice, which included a dye-swap for each Btk-/WT combination to compensate for possible dye-specific effects on homing capacity. The ratio of the input population was determined and normalized to 1 to correct the homing ratio (Btk-deficient cells/ WT cells).

**Immunoprecipitation and Immunoblotting**

For analysis of Btk and PLCγ2 tyrosine phosphorylation, 1.5-3 x 10^7 cells, serum starved for 1 hr at 37°C (39.5°C for DT40), were stimulated with 200 ng/ml SDF-1 or 10 μg/ml anti-IgM and immunoprecipitated, essentially as described (van der Voort et al., 1999). Briefly, the cells were lysed by addition of an equal volume ice-cold 2 x lysis buffer (20 mM Tris-HCl pH 8, 300 mM NaCl, 2% Nonidet P-40, 20% glycerol, 10 mM EDTA, 4mM Na3VO4, 10 mM NaF, and 2 EDTA-free protease inhibitor cocktail tablets (Roche) per 50ml). After preclearance with protein A-Sepharose beads (Pharmacia), the lysates were incubated with 1-3 μg anti-Btk (C20), anti-T7, anti-P-Btk, anti-PLCγ2 (Q20), or anti-phosphotyrosine (PY20 or 4G10) antibody at 4°C O/N, and immunoprecipitation was carried out by adding protein A- or G-Sepharose beads for 30 min. Immunoprecipitates and total lysates were analyzed by SDS-PAGE and immunoblotting, using anti-P-Btk, anti-Btk (G149-11), anti-P-PLCγ2, anti-PLCγ2 (H160), or antiphosphotyrosine (4G10). Please note that none of the tested Btk or PLCγ2 antibodies could immunoprecipitate Btk or PLCγ2 from (chicken) DT40 cells with sufficient efficiency for phosphorylation analysis. Therefore, Btk-deficient DT40 cells reconstituted with T7-tagged Btk were used for the Btk phosphorylation studies in DT40 cells. For analysis of ERK and PKB phosphorylation, after stimulation of 10^7 cells /ml RPMI with 100 ng/ml SDF-1, cells were directly lysed in SDS-PAGE sample buffer. 2 x 10^5 cells were applied on a 10% SDS-PAGE gel and immunoblotted with anti-phospho-MAPK1/2 and anti-phospho-PKB, as described (Spaargaren et al., 2003).

**Rac and Rap1 Activity Pull-Down Assays**

Cells were resuspended to 0.8 x 10^7 cells/0.4 ml RPMI and stimulated with SDF-1 (100 ng/ml). Reactions were terminated by adding 0.4 ml of cold 2 x lysis buffer (100 mM Tris-HCl pH 7.4, 400 mM NaCl, 5 mM MgCl2, 2% Nonidet P-40, 20% glycerol, 2 x
EDTA-free protease inhibitor cocktail 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 a 20% solution per sample) were pre-coupled with GST-RalGDS-RBD or GST-PAK-RBD fusion protein by continuous mixing for 30 minutes at 4°C with bacterial cell lysates from *E. coli* strain BL21 transformed with pGEX4T-RalGDS-RBD96 or pGEX2T-PAK-RBD. After being washed three times with cell lysis buffer, these pre-coupled 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 15% SDS-PAGE, and immunoblotted with anti-Rap1 or anti-Rac1. pGEX4T-RalGDS-RBD96, encoding a GST fusion protein containing the 96 AA Rap and Ras binding domain of RalGDS (AA 789-884) was generated by subcloning a 300 bp EcoRI-AvaII (blunted) fragment from pGAD-RalGDS-RBD (Spaargaren and Bischoff, 1994) into EcoRI- and Smal-digested pGEX4T3. The pGEX2T-PAK-RBD plasmid containing the Rac binding domain of PAK (AA 56-272) was kindly provided by Dr. J. Collard (Netherlands Cancer Institute, Amsterdam, the Netherlands).

**Statistical Analysis**

The unpaired two-tailed Student’s t-test was used to determine the significance of differences between means. Unless otherwise indicated, all relevant comparisons (e.g., control versus inhibitors or WT versus gene-deficient cells) were significantly different (*p*<0.05).
Supplemental References


