B Cell Antigen Receptor Engagement Inhibits Stromal Cell-derived Factor (SDF)-1α Chemotaxis and Promotes Protein Kinase C (PKC)-induced Internalization of CXCR4

By Rodolphe Guinamard,* Nathalie Signoret,† Masamichi Ishiai,§ Mark Marsh,‡ Tomohiro Kurosaki,§ and Jeffrey V. Ravetch*

From the *Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York 10021; the †Medical Research Council Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, London WC1E 6BT, United Kingdom; and the §Department of Molecular Genetics, Kansai Medical University, Moriguchi, Osaka 570, Japan

Summary

The entry of B lymphocytes into secondary lymphoid organs is a critical step in the development of an immune response, providing a site for repertoire shaping, antigen-induced activation and selection. These events are controlled by signals generated through the B cell antigen receptor (BCR) and are associated with changes in the migration properties of B cells in response to chemokine gradients. The chemokine stromal cell–derived factor (SDF)-1α is thought to be one of the driving forces during those processes, as it is produced inside secondary lymphoid organs and induces B lymphocyte migration that arrests upon BCR engagement. The signaling pathway that mediates this arrest was genetically dissected using B cells deficient in specific BCR-coupled signaling components. BCR-induced inhibition of SDF-1α chemotaxis was dependent on Syk, BLNK, Btk, and phospholipase C (Plc)γ2 but independent of Ca2+ mobilization, suggesting that the target of BCR stimulation was a protein kinase C (PKC)-dependent substrate. This target was identified as the SDF-1α receptor, CXCR4, which undergoes PKC-dependent internalization upon BCR stimulation. Mutation of the internalization motif SSXXIL in the COOH terminus of CXCR4 resulted in B cells that constitutively expressed this receptor upon BCR engagement. These studies suggest that one pathway by which BCR stimulation results in inhibition of SDF-1α migration is through PKC-dependent downregulation of CXCR4.

Key words: chemokine • lymphocyte • migration • signaling • phospholipase C

The ability to successfully mount an immune response depends on the interaction of multiple cell types, insuring that autoreactivity is avoided, diversity and specificity are achieved, and a memory of the encounter is established. The anatomic compartmentalization of APCs, T and B lymphocytes, and effector cells in secondary lymphoid organs like the spleen or lymph node maximizes the probability of interaction of the cellular and humoral factors necessary to achieve these responses. Zones containing specific cellular populations allow ordered and sequential interactions to take place, thereby insuring that only a small subset of B lymphocytes are stimulated and produce antibody or undergo further somatic hypermutation within germinal centers (GCs). Although important during an immune response, changes in the migration and position of B cells inside lymphoid organs are also intimately associated with their stage of maturation. An antigen receptor (AgR)-dependent positive selection process allows some of the newly generated bone marrow B cells to progress to the mature B cell stage. This selection event is associated with follicular recolonization. In those sites, in contact with follicular dendritic cells, B lymphocytes might receive survival signals and become recruited to the long-lived B cell repertoire.

Above a threshold of AgR engagement, B cells migrate toward or arrest within the periarteriolar lymphoid sheath, where the probability of encountering Ag-specific T cells is maximal. In the absence of cognate interactions with T cells, activated B cells will die, and as a consequence, strong autoreactivity will be purged from the repertoire. R are activated B cells that receive cognate T cell help will proliferate, and some of them will join follicles to form GCs. The GC reaction generates B cells with new migration properties. Thus, plasmocytes can relocate into the red pulp of the spleen, join the peripheral circulation, and enter the bone marrow, where they will produce antibody for an extended period of time.
circulate or reside in the marginal zone of the spleen, the privileged area for reencounter with blood-borne antigen (1).

Four chemokines with the ability to direct the migration of B lymphocytes are known to be expressed within secondary lymphoid organs. These are B lymphocyte chemotactic factor (BLT1) or B cell-attaining chemokine (BAC-1), which binds to the Burkitt’s lymphoma receptor 1 (BLR1) (or CXCR5) (9, 10); secondary lymphoid tissue chemokine (SLC, or 6C-kine, exodus-2) and Epstein-Barr virus-induced molecule 1 ligand chemokine (ELC, or MIP3b), both binding to the chemokine receptor CCR7 (11, 12); and SDF-1α (or pre-B cell growth-stimulating factor [PBSF]), which stimulates cells through CXCR4 (13, 14). The importance of these chemokines in the migration and selection processes is suggested either by the differential expression of their receptors during B cell maturation or by the fact that AgR engagement can modulate the associated chemotactic responses. BLR1, the receptor for BLC, is only expressed when cells mature from newly generated to follicular B cells; this expression is likely to account, at least in part, for the tropism of these cells for follicles. Thus, inactivation of BLR1 by targeted gene disruption is associated with deficits in spleen and Peyer’s patch follicles (15, 16). BCR activation has a direct impact on B cell chemotaxis to ELC and SDF-1α, resulting in either enhancement or arrest, respectively (12, 14). Given that AgR signaling controls B cell maturation and is associated with cell relocalization, the direct regulation of chemokine responsiveness by BCR engagement is likely to play a major role in driving the selection and organization of B lymphocytes within lymphoid organs.

The mechanism by which BCR ligation may lead to SDF-1α unresponsiveness has been addressed in this study by using the genetically defined DT40 B cell system. Targeted disruption of many of the signaling components of the BCR-stimulated pathway have been generated in these cells and have demonstrated great utility in defining the mechanisms by which antigen stimulation of B cells results in cellular activation. DT40 B cells migrate efficiently to SDF-1α and are arrested in their migration by BCR cross-linking. Through the analysis of a series of signaling mutants of DT40 cells, we have established that BCR stimulation results in a calcium-independent, protein kinase C-dependent downregulation of the SDF-1α receptor CXCR4. These studies suggest mechanisms by which diverse signals may influence this pathway and thereby modulate redirected migration of B cells inside lymphoid tissues.

Materials and Methods

Reagents. Human SDF-1α, 12G5 anti-CXCR4, and anti-SDF-1α antibodies were from R & D Systems Inc.; human serum albumin (HSA) and BSA, FITC-conjugated F(ab’)2 fragment of goat anti-mouse IgG, and PMA were from Sigma Chemical Co. The M4 mAb anti-chicken IgM has previously been described (17).

DT40 Cell Culture and Transfections. Wild type (wt) and Btk−/−, Syk−/−, phospholipase C (PLC)-γ2−/−, BLNK−/−, or IP3R−/−-deficient chicken DT40 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1% chicken serum, 50 mM 2-ME, 2 mM L-glutamine, and antibiotics. The constructions containing wt and SSLKIL→AALKAA (4A) mutants of human CXCR4 have been described previously (23). Cells were transfected by electroporation at 250 V and 960 μF in PBS (107 cells/0.5 ml). 20 μg expression constructs were cotransfected with 2 μg pBabe-puroV vector (24). Transfectants were selected in 0.5 μg/ml puromycin 24 h after electroporation. The presence of CXCR4 surface expression was determined by FACS® analysis with 12G5 mAb and FITC-conjugated secondary antibody. In each condition: DT40-wt + CXCR4 (wt or 4A), Plgc2−/− + CXCR4 (wt or 4A). Two clones were analyzed for the experiments; they had comparable and homogenous levels of expression ranging from 120 to 200 arbitrary units (data not shown).

Chemotaxis Assay. DT40 cells (105 cells per condition) were washed and resuspended in 100 ml RPMI 1640 and 0.25% HSA and incubated for 1 h at 37°C in the presence of different concentrations of anti-BCR antibodies. Cells were then added to the top chamber of a 6.5 mm-diameter, 5-mm pore polycarbonate transwell culture insert (Costar Corp.); the lower chamber contained RPMI 0.25% HSA alone or supplemented with 100 nM SDF-1α. Migration proceeded for 3 h at 37°C. Transmigrated cells were then vigorously suspended and counted with a FACScan™ (Becton Dickinson) for 20 s at 60 μl/min, with gating on forward and side scatter to exclude debris. 100% migration was obtained by counting cells added directly to the lower chamber.

CXCR4 Surface Expression Analysis. Cells expressing wt or the 4A mutant of human CXCR4 were resuspended in RPMI 1640 and 0.25% HSA at 105 cells/ml. They were then diluted twice with the same buffer or with medium supplemented with 200 nM SDF-1α, 200 nM PMA, or 20 μg/ml anti-BCR mAb and kept either at 4°C (for T = 0) or incubated at 37°C for 1 or 2 h. All subsequent steps were carried out at 4°C. Cells were washed once in staining buffer (PBS, 0.5%MCA, 0.05% azide, and 5% normal goat serum) and incubated in the presence of 12G5 anti-CXCR4 antibodies for 1 h. After two washes, primary antibodies were detected using a FITC-conjugated F(ab’)2 fragment of goat anti-mouse IgG. Signals were acquired on a FACScan™. R results are given as percentage of controls 100% corresponding to cells incubated in medium alone. No inhibition of 12G5 binding was found when cells were preincubated with SDF-1α, PMA, or anti-BCR at 4°C, showing that modulation of 12G5 binding was the consequence of an active process. Further controls included absence of staining of nontransfected cells by 12G5 mAb (data not shown) or of CXCR4-transfected cells by an isotype control primary antibody (Fig. 3 B).

Results. DT40 B cells migrate in response to SDF-1α and A on BCR Ligation. To determine if the chicken B cell line DT40 was an accurate model for SDF-1α-dependent migration and BCR-induced arrest, we characterized the ability of these cells to migrate in response to this chemokine. DT40 cells were placed on the upper side of a transwell apparatus, and human SDF-1α was placed on the opposite side. DT40 cells migrated efficiently (Fig. 1) to this chemokine. The migration was specifically inhibited by anti-SDF-1α antibody but not by an irrelevant antibody (not shown), demonstrating the specificity of this migration effect. Cross-linking of the BCR on DT40 cells with the murine mAb M4 for 1 h resulted in a dose-dependent inhi-
tracellular calcium release in response to BCR-induced IP$_3$ activation. IP$_3$-stimulated release of calcium from intracellular stores in response to IP$_3$ is strongly dependent upon PKC activation through Plc$_{2,3}$-dependent internalization of the CXCR4 pathway is present downstream of CXCR4, as CXCR4, is rapidly internalized upon activation of PKC by phorbol esters or SDF-1$_{1\alpha}$ exposure (Fig. 3). However, BCR-induced downregulation of CXCR4 is blocked in the Plc$_{2}$-deficient DT40 background, which correlates with the inability of this mutant to display BCR-mediated arrest of SDF-1$_{1\alpha}$ migration. To determine if Plc$_{2}$ is upstream, downstream, or pleiotropic in relation to CXCR4, this mutant was tested for its ability to respond to phorbol esters or SDF-1$_{1\alpha}$ exposure (Fig. 3), indicating that Plc$_{2}$ lies upstream of CXCR4 in the BCR-induced internalization of the CXCR4 pathway.
1464 B Cell Antigen Receptor–induced Internalization of CXCR4

pathway and that SDF-1α-induced internalization is independent of Plcγ2 activation. These results thus suggest that the BCR-induced arrest of SDF-1α-directed migration may be due in part to CXCR4 internalization triggered by BCR-mediated stimulation of Plcγ2 and PKC activation. In addition, they show that SDF-1α and BCR activation lead to CXCR4 surface downregulation through different pathways in DT40 B cells.

The SSXXIL motif in the COOH Terminus of CXCR4 is required for BCR-induced Receptor Internalization. Signoret et al. (23) have demonstrated that a SSXXIL motif, similar to that required for endocytosis of CD4 and the TCR complex, is required for phorbol ester–induced, but not ligand-induced, internalization of CXCR4. To determine the contribution of this motif to the BCR-induced internalization of CXCR4 in DT40 cells, we generated stable transfectants of DT40 wt or Plcγ2-deficient cells expressing a CXCR4 mutant in which the SSLKIL sequence was replaced by AALKAA. As seen in Figs. 3 and 4, wt DT40 cells expressing the wt human CXCR4 receptor internalize this receptor in response to BCR cross-linking, SDF-1α treatment, and PMA stimulation. In contrast, the SSLKIL→AALKAA mutant CXCR4 receptor (designated 4A in Fig. 4), whether expressed in wt or Plcγ2-deficient DT40 cells, was incapable of BCR- or PMA-induced internalization (Fig. 4) but retained significant receptor downmodulation in response to SDF-1α. The 4A mutant CXCR4 was unable to migrate in response to SDF-1α, either in the presence or absence of BCR cross-linking (data not shown). The basis for this migration defect has not been determined. BCR- and Plcγ2-dependent internalization of CXCR4 thus appears to utilize the same pathway as PMA, a PKC-dependent downmodulation of this receptor.

Discussion

AgR signaling determines B cell maturation, selection, and orientation within lymphoid organs. Progression from newly generated B cells into MZ and follicular B cells is driven by BCR signaling and is associated with specific anatomic localization inside the spleen. Supra-threshold AgR engagement redirects B cells from follicles, MZ, or blood circulation toward the periarteriolar lymphoid sheath. Depending on their ability to direct cognate interaction with T cells, a humoral response will emerge or B cells will die in a few days (2, 5, 6, 27).

It is now clearly established that chemokines play an important role in these relocalization processes. Thus, the expression of BLR1 is associated with follicular B cell maturation and is required for their tropism in the spleen and Peyer’s patch, whereas SDF-1α and SLE responses are rapidly regulated upon BCR engagement (12, 14, 16). The BCR-induced downregulation of CXCR4 demonstrated here offers a first example in which differential AgR engagement might promote differential responsiveness to a
chemokine and allow repertoire-based interclonal competition for migration toward a restricted, chemokine-secreting environment (28). However, as seen in Fig. 3, BCR cross-linking results in a twofold reduction in CXCR4 expression. Although this change in expression may account for some of the migration inhibition seen, it suggests that other pathways may be involved as well. The generation of CXCR4 mutants that are deficient in BCR-induced downregulation yet retain chemotactic response to SDF-1α will allow further dissection of the contribution of this pathway to the antigen-driven compartmentalization of lymphocytes. In addition, the definition of SDF-1α secretion sites will provide important clues toward the understanding of B cell migration and selection.

In vitro studies have shown that pro-B cells are dependent on contact with stromal cells and cytokines for survival, whereas cells expressing the pre-B cell receptor are only dependent on soluble factors (29, 30). Bone marrow stromal cells produce SDF-1α, and pro-B cells respond to this chemokine (13, 31). It is tempting to transpose our data from the early steps of pro-B to pre-B cell transition. Thus, like BCR, pre-BCR signaling might induce the downregulation of CXCR4 and block SDF-1α–dependent migration of pre-B cells toward stromal cells. Therefore, CXCR4 downregulation might allow B cells to lose stromal cell tropism upon successful rearrangement of their IgH gene and signaling through the pre-B cell receptor. This mechanism could guarantee the restriction of rare niches to pro-B cells. In agreement with the importance of SDF-1 during early B cell differentiation, SDF-1 and CXCR4 knockout mice show a profound defect in pro-B cell production (32–34). The present definition of a pathway from BCR to the CXCR4 receptor and of a motif responsible for this coupling may allow the construction of mutants to directly test the role of this pathway in vivo. Such analysis might provide insights that will define how Ag-dependent competitive migration participates in B cell maturation and response to Ag.

This work was supported in part by grants from the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIH); the National Institute of Allergy and Infectious Diseases, NIH; the Irvington Institute for Immunological Research; and the Association pour la Recherche contre le Cancer.

Address correspondence to Jeffrey V. Ravetch, Laboratory of Molecular Genetics and Immunology, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: 212-327-7321; Fax: 212-327-7319; E-mail: ravetch@rockvax.rockefeller.edu

Received for publication 18 February 1999 and in revised form 15 March 1999.

References

1. Liu, Y.J., J. Zhang, P.J. Lane, E.Y. Chan, and I.C. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur. J. Immunol. 21:2951–2962.

2. MacLennan, I.C., Y.J. Liu, and G.D. Johnson. 1992. Maturation and dispersal of B-cell clones during T cell-dependent antibody responses. Immunol. Rev. 126:143–161.

3. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. J. Exp. Med. 173:1357–1371.

4. Lorton, J., B. A. Roobottom, S. Oldfield, and I.C. MacLennan. 1987. Newly produced virgin B cells migrate to secondary lymphoid organs but their capacity to enter follicles is restricted. Eur. J. Immunol. 17:1311–1316.

5. Goodnow, C.C., J.G. Cyster, S.B. Hartley, S.E. Bell, M.P. Cooke, J.L. Healy, S. Akkaraju, J.C. Rathamel, S.L. Pogue, and K.P. Shokat. 1995. Self-tolerance checkpoints in B lymphocyte development. Adv. Immunol. 59:279–368.

6. Fulcher, D.A., A.B. Lyons, S.L. Korn, M.C. Cook, C. Koleda, C. Parish, D.S. Fazekas, and A. Besten. 1996. The fate of self-reactive B cells depends primarily on the degree of antigen receptor engagement and availability of T cell help. J. Exp. Med. 183:2313–2328.

7. Cyster, J.G., S.B. Hartley, and C.C. Goodnow. 1994. Competition for follicular niches excludes self-reactive cells from the recirculating B-cell repertoire. Nature 371:389–395.

8. Slifka, M.K., R. Antia, J.K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. Immunity. 8:363–372.

9. Gunn, M.D., V.N. Ngo, K.M. Ansel, E.H. Ekland, J.G. Cyster, and L.T. Williams. 1998. A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. Nature 391:799–803.

10. Legler, D.F., M. Loetscher, R.S. Roos, I. Clark-Lewis, M. Baggioili, and B. Moser. 1998. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. J. Exp. Med. 187:655–660.

11. Gunn, M.D., K. Tangemann, C. Tam, J.G. Cyster, S.D. Rosen, and L.T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. Nature 391:799–803.

12. Ngo, V.N., H.L. Tang, and J.G. Cyster. 1998. Epstein-Barr virus–induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. J. Exp. Med. 188:181–191.

13. Bleul, C.C., R.C. Fuhlbrigge, J.M. Casasnovas, A. Aiuti, and T.A. Springer. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell–derived factor 1 (SDF-1). J. Exp. Med. 184:1101–1109.
14. Bleul, C.C., J.L. Schultze, and T.A. Springer. 1998. B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. J. Exp. Med. 187:753–762.

15. Schmidt, K.N., C.W. Hsu, C.T. Griffin, C.C. Goodnow, and J.G. Cyster. 1998. Spontaneous follicular exclusion of SHP-1-deficient B cells is conditional on the presence of competitor wild-type B cells. J. Exp. Med. 187:929–937.

16. Forster, R., A.E. Matis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell. 87:1037–1047.

17. Ono, M., H. Okada, S. Bolland, S. Yanagi, T. Kurosaki, and J.V. Ravetch. 1997. Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling. Cell. 90:293–301.

18. Takata, M., and T. Kurosaki. 1996. A role for Brutons tyrosine kinase in B cell antigen receptor-mediated activation of phospholipase C-γ2. J. Exp. Med. 184:31–40.

19. Kurosaki, T., S.A. Johnson, L. Pao, K. Sada, H. Yamamura, and J.C. Cambier. 1995. Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J. Exp. Med. 182:1815–1823.

20. Takata, M., Y. Homma, and T. Kurosaki. 1995. Requirement of phospholipase C-γ2 activation in surface immunoglobulin M–induced B cell apoptosis. J. Exp. Med. 182:907–914.

21. Ishii, M., M. Kurosaki, R. Pappu, K. Oikawa, I. R Onko, C. Fu, M. Shibata, A. Iwamatsu, A.C. Chan, and T. Kurosaki. 1999. BLNK required for coupling Syk to PLC-γ2 and Rac1-JNK in B cells. Immunity. 10:117–126.

22. Sugawara, H., M. Kurosaki, M. Takata, and T. Kurosaki. 1997. Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-triphosphate receptors in signal transduction through the B-cell antigen receptor. EMBO J. (Eur. Mol. Biol. Organ.) 16:3078–3088.

23. Signoret, N., M.M. Rosenkilde, P.J. Klasse, T.W. Schwartz, M.H. Malim, J.A. Hoxie, and M. Marsh. 1998. Differential regulation of CXCR4 and CCR5 endocytosis. J. Cell Biol. 139:2819–2830.

24. Morgenstern, J.P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. 18:3587–3596.

25. Amara, A., S.L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggioni, J.L. Virelizier, and F. Azenzana-Seisdedos. 1997. HIV coreceptor downregulation as antiviral principle: SDF-1α-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J. Exp. Med. 186:139–146.

26. Chen, X., F. Martin, K.A. Forbush, R.M. Perlmuter, and J.F. Kearney. 1997. Evidence for selection of a population of multi-reactive B cells into the splenic marginal zone. Int. Immunol. 9:27–41.

27. Goodnow, C.C., and J.G. Cyster. 1997. Lymphocyte homing: the scent of a follicle. Curr. Biol. 7:R19–R222.

28. Holmes, W. Dallas, et al. 1997. Phorbol esters and SDF-1 induce rapid endocytosis and downmodulation of the chemokine receptor CXCR4. J. Cell Biol. 139:651–664.