$P < 0.01$; r, correlation coefficient.
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27. We obtained fish and invertebrates from these three lakes by netting or dredging in June to August of 1992 and 1993. Samples were frozen shortly after collection and kept at −40°C until analyzed for toxaphene and $\delta^{15}N$ according to the methods in (*15*). There were no significant differences between the slopes of the regressions with the use of analysis of covariance ($P < 0.05$). Similar relations between $\delta^{15}N$ and other persistent chlorinated organics were also found (*15*).
28. Tukey multiple comparison (lake trout and lake whitefish) and Student's t tests (burbot) were used to test for significant differences. Within species, bars with the same letter were not significantly different ($P < 0.05$).
29. Research was supported by the Arctic Environmental Strategy Northern Contaminants Program (Indian and Northern Affairs, Canada), the Arctic Institute of North America Fellowship (Alberta), a Canadian Circumpolar Institute Boreal Alberta research grant (Alberta), the Northern Research Institute Fellowship (Yukon), a Northern Scientific Training Program grant (Indian and Northern Affairs, Canada), a Natural Science and Engineering Research Council (Canada) operating grant to D.W.S., and a Tri-Council Eco-Research doctoral fellowship to K.A.K. We thank B. Billeck, L. Chin, B. Danell, D. Davidge, N. deGraff, J. Eamer, T. Genier, S. Kidd, S. Lamontagne, B. Lendrum, S. Lendrum, D. Metner, A. Omelchenko, M. Palmer, B. Parker, G. Pope, S. Thompson, A. von Finster, P. Wilkinson, the Department of Fisheries and Oceans (Yukon), the Department of Renewable Resources (Yukon Government), Environment Canada (Yukon), Ta'an Kwach'an First Nation, and the Yukon Contaminants Committee for assistance. We thank M. Alaee (Environment Canada, Burlington, Ontario) for providing water extracts; R. Currie, B. Grift, K. Koczanski, S. Mohammed, P. Ramlal, B. Rosenberg, and G. Stern for laboratory analyses; and B. Miskimmin for reviewing an earlier draft of the manuscript.

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A Role in B Cell Activation for CD22 and the Protein Tyrosine Phosphatase SHP

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CD22 is a membrane immunoglobulin (mIg)–associated protein of B cells. CD22 is tyrosine-phosphorylated when mIg is ligated. Tyrosine-phosphorylated CD22 binds and activates SHP, a protein tyrosine phosphatase known to negatively regulate signaling through mIg. Ligation of CD22 to prevent its coaggregation with mIg lowers the threshold at which mIg activates the B cell by a factor of 100. In secondary lymphoid organs, CD22 may be sequestered away from mIg through interactions with counterreceptors on T cells. Thus, CD22 is a molecular switch for SHP that may bias mIg signaling to anatomic sites rich in T cells.

The diverse array of antigen receptors on B and T lymphocytes, (mIgs and T cell receptors, respectively) can bind an almost unlimited number of different antigens. Recombination among the genetic elements that encode these receptors accounts for this diversity, but it does so without reference to potential antigens and creates receptors that are potentially self-reactive. The solution to this problem, as first suggested by Bretscher and Cohn (*1*), is to require a second signal for the clonal expansion and differentiation of antigen-specific B cells into plasma cells. This second signal is provided by helper T cells in the form of gp39 (*2*), the ligand for CD40, during its cognate interaction with the antigen-reactive B cell. In the absence of a second signal, the first signal of mIg induces tolerance or death of the B cell (*3*). There is a potential difficulty with this strategy: the low frequency of primed, antigen-specific helper T cells. This problem may be resolved by restriction of the activation of B cells by antigens to secondary lymphoid organs. T cells recirculate through these structures in large numbers, thus increasing the potential for encounters between antigen-specific B and T cells. To date, no mechanism has been described that could provide this level of control.

CD22 is a membrane protein (*4*) that may enable the B cell to sense the presence of adjacent lymphocytes and regulate signaling by mIg. The NH_2-terminal Ig-like domains of the extracellular region of CD22 (*5*) have specificity for glycoconjugates containing α 2,6–linked sialic acid that are expressed preferentially by B and T cells (*6*). CD22 associates with mIg, and its intracellular domain is tyrosine-phosphorylated after ligation of mIg (*7*), enabling interaction with phosphotyrosine-specific SH2 domains of intracellular signaling proteins.

The protein tyrosine phosphatase (PTP) SHP (also termed PTP1-C, SHPTP1, and HCP) contains two SH2 domains (*8*). The *SHP* gene is mutated in *motheaten* (*me*) and *viable motheaten* (*me^v*) mice (*9*) that have an expanded B-1 subset of B cells, elevated concentrations of immunoglobulin M (IgM) and IgG3 (*10*), and autoimmune arthritis and glomerulonephritis. Recently, *me^v* B cells were shown to induce the release of intracellular calcium in response to lower concentrations of antigen than did normal B cells (*11*).

We determined whether tyrosine-phosphorylated CD22 interacts with SHP by precipitating the PTP from NP-40 lysates of Daudi B lymphoblastoid cells that were resting or had been activated by ligating mIgM (*12*, *13*). The proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and immunoblotted sequentially with antibody to phosphotyrosine and antibody to SHP. In resting Daudi cells, SHP was not tyrosine-phosphorylated, and it coimmunoprecipitated a phosphorylated protein of approximately 130 kD that was barely detectable (Fig. 1A). In activated cells, the PTP was tyrosine-phosphorylated and was associated with phosphorylated proteins of 120 to 130 kD and 72 kD. We immunoprecipitated CD22 from replicate samples of the Daudi cells and subjected the precipitates to the same immunoblot analysis. The tyrosine-phosphorylated triplet of CD22 comigrated with the SHP-associated triplet at 120 to 130 kD. The increase in phosphorylation of CD22 induced by mIgM was associated with an increase in the amount of coimmunoprecipitating SHP (Fig. 1B). The SHP that was associated with CD22 appeared to be relatively less phosphorylated than the total SHP, which suggests that it was the tyrosine phosphorylation of CD22, rather than of SHP, that led to the binding of the PTP.

To determine which of the six tyrosines of the cytoplasmic domain of CD22 mediate the interaction with SHP, murine splenic B cells were permeabilized and incubated with buffer alone or buffer containing each of six phosphotyrosyl peptides corresponding to the sequence of

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murine CD22 (*5*, *14*). After treatment with pervanadate to induce phosphorylation of CD22, lysates were prepared and the CD22 was immunoprecipitated. Immunoblots of the resolved immunoprecipitates were probed with antibody to SHP. SHP associated with CD22 in the pervanadate-treated cells, and this association was inhibited by three tyrosine-phosphorylated peptides, m2*, m5*, and m6*, which contain the sequences PO_4-YAIL, PO_4-YSEL, and PO_4-YVTL (*15*), indicating that a motif recognized by the SH2 domains is PO_4-YXXL (*15*) (Fig. 2A). The human peptides corresponding to the m2*, m5*, and m6* phosphotyrosyl peptides also inhibited the coimmunoprecipitation of SHP with CD22 from NP-40 lysates of human B lymphoblastoid cells that had been activated through mIgM.

Recombinant SHP lacking its SH2 domains possesses increased phosphatase activity (*16*). We investigated whether the negative regulatory effect of these domains could be overcome by the CD22-related phosphotyrosyl peptides. A glutathione-S-transferase (GST) fusion protein (*17*) having the full-length enzyme was incubated with the synthetic phosphotyrosyl substrate [^{32}P]Raytide (Oncogene Science, Uniondale, NY) (*8*) alone and in the presence of increasing concentrations of the peptides. The PTP activity of SHP was increased in a dose-related manner by the same three phosphotyrosyl peptides m2*, m5*, and m6* that competed with CD22 for interaction with SHP (Fig. 2B). The nonphosphorylated forms of m2, m5, and m6 were ineffective. The tyrosine-phosphorylated human CD22 homologs of these peptides (*14*), Y(762)TTL, Y(822)SEL, and Y(842)VIL (*15*), also increased the activity of SHP.

These findings suggested a means by which CD22 could have an inhibitory feedback effect on the activation of the B cell: Ligation of mIg induces the tyrosine phosphorylation of CD22, which can then localize and activate SHP at the intracellular site of the signaling complex. This function of CD22 would be dependent on its ability to coaggregate with ligated mIg. Therefore, we investigated whether restricting the lateral diffusion of CD22 in the plane of the membrane would enhance the response of B cells to the ligation of mIg. Purified, high-density, human tonsillar B cells (*18*) were incubated with incremental concentrations of monoclonal antibody to IgM and IL-4 alone, or in the presence of beads coated with antibody to CD22, with isotype-matched IgG2b of irrelevant specificity, or with antibody to CD19 as a control for the effects of adhesion of beads to the B cells (*19*). Within 1 hour of incubation, B cells became coated with beads bearing antibody to CD22 or antibody to CD19 but not the control IgG2b. The presence of the beads bearing antibody to CD22, but not of the beads bearing antibody to CD19 or control IgG2b, lowered the concentration of antibody to IgM that was required to achieve the maximal amount of DNA synthesis by two orders of magnitude (Fig. 3).

To determine whether the sequestration of CD22 from mIgM altered the pattern of tyrosine phosphorylation, we incu-

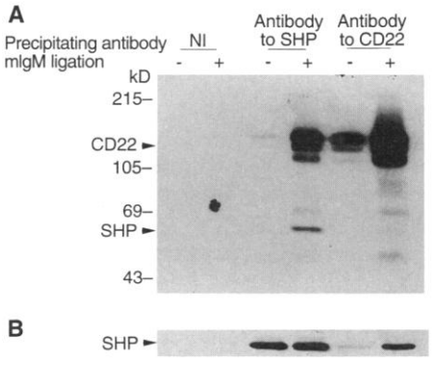

Fig. 1. Association of CD22 with SHP in B lymphocytes stimulated through mIgM. Replicate samples of 5 × 10^7 Daudi B lymphoblastoid cells were incubated for 5 min at room temperature with buffer alone or with buffer containing goat F(ab')$_2$antibody to IgM (10 μg/ml) (*12*). The cells were lysed in NP-40 (*13*), and immunoprecipitates were prepared with rabbit antibody to SHP and monoclonal mouse antibody to CD22, respectively, and with a mixture of nonimmune (NI) rabbit serum and an isotype-matched monoclonal antibody (*12*). The precipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and sequentially probed with ^{125}I-labeled 4G10 antibody to phosphotyrosine (*12*) (**A**) and polyclonal antibody to SHP (*12*) (**B**), the binding of which was detected by ECL.

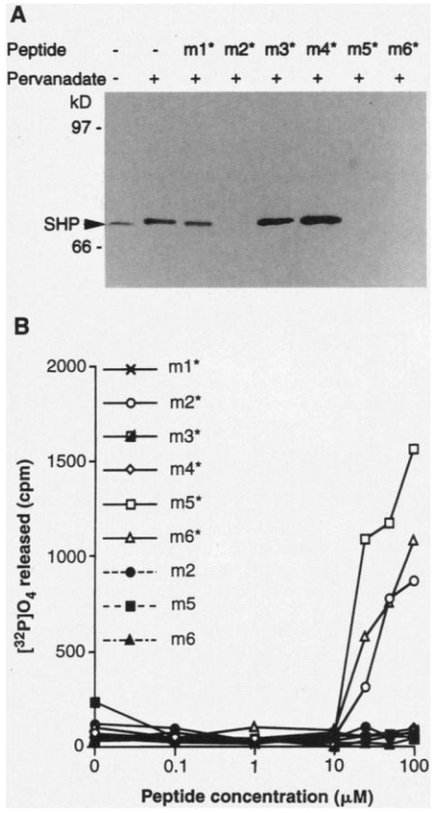

Fig. 2. Interaction of CD22 phosphotyrosyl-peptides with SHP. (**A**) Inhibition by peptides of coimmunoprecipitation of CD22 and SHP. Replicate samples of 3 × 10^7 resting murine splenic B cells were permeabilized and incubated with 100 μM of each of six phosphotyrosyl peptides (*14*) corresponding to the sequence of murine CD22 (*4*): m1*, QGCY*(773)NPAMDD; m2*, TVSY*(783)AILRFP; m3*, DTVTY*(817)SVIQK; m4*, MGDY*(828)ENVNPS; m5*, SIHY*(843)SELVQF; and m6*, EDVDY*(863)VTLKH. After treatment with 100 μM pervanadate for 10 min, the cells were lysed with digitonin. Lysates were precleared with an irrelevant IgG2b, RG7/9.1; coupled to Sepharose; and then immunoprecipitated with CY34.1.2 antibody to CD22 (*12*), also coupled to Sepharose. Eluates were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit antibody to SHP. (**B**) Activation of SHP by peptides. The GST-SHP fusion protein (*17*) was incubated for 10 min with [^{32}P]Raytide alone and in the presence of incremental concentrations of each of the six phosphotyrosine-containing CD22-related peptides (m1* to m6*) and of three peptides lacking phosphotyrosine (m2, m5, and m6, respectively). Release of [^{32}P]O_4 was assayed (*8*).

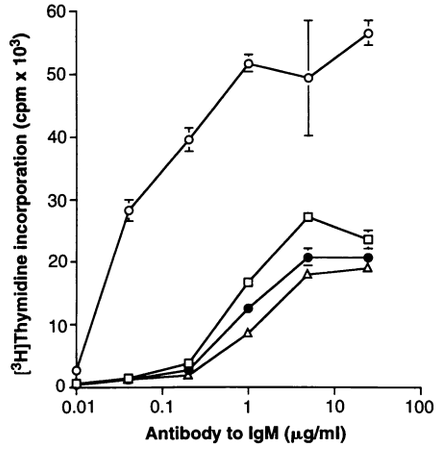

Fig. 3. Effect of immobilization of CD22 on DNA synthesis in human B lymphocytes stimulated by ligating mIgM. Replicate samples of 1 × 10^5 purified, high-density, tonsillar B cells (*18*) were preincubated for 1 hour at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum (squares) or containing beads bearing IgG2b of irrelevant specificity (solid circles), B3 IgG2b antibody to CD22 (open circles), or B4 IgG2b antibody to CD19 (triangles) (*12*, *18*). Incremental amounts of DA4.4 antibody to IgM were added, and incubation continued in the presence of recombinant Il-4 (200 U/ml) (Genzyme) for 84 hours. Cultures were pulsed with [^{3}H]thymidine for the last 16 hours. Results are the means ± SDs of triplicates.

bated purified, high-density, tonsillar B cells with antibody to IgM and IL-4 in the presence of beads coated with antibody to CD22 or with control IgG2b. The cells were lysed after timed intervals, and the lysates were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine. The beads bearing antibody to CD22 did not induce tyrosine phosphorylation alone but increased the phosphorylation of a 90-kD protein after stimulation of the cells with antibody to IgM for 1, 5, and 10 min, respectively (Fig. 4). A modest increase in the phosphorylation of a 100-kD protein occurred in the presence of the beads bearing antibody to CD22 and of antibody to IgM, whereas that of a 140-kD protein was slightly decreased. Increased phosphorylation of the 90-kD protein in the presence of antibody to IgM and the beads bearing antibody to CD22 was observed in two additional experiments.

The physiologically relevant analog of beads bearing antibody to CD22 may be the B and T cells that surround antigen-stimulated B cells in secondary lymphoid organs. If so, the threshold at which a B cell responds to antigen will be lower within a lymphoid microenvironment. Such an environment would provide the secondary signals from T cells that maintain the activation of B cells after ligation of mIg.

CD22 is the third membrane protein that modulates the signaling threshold of B cells in a phosphotyrosine-dependent manner, the others being CD19 and FcγRIIB. CD19 binds the positive regulator of signal transduction, phosphatidylinositol 3-kinase (*20*), and amplifies signaling when co-ligated to mIg (*18*). FcγRIIB also mediates its effect when co-ligated to mIg but has an opposing effect of suppressing signaling. The effect is mediated by the negative regulator SHP, which has bound to the phosphorylated receptor (*21*). CD22 resembles FcγRIIB in its interaction with SHP, but differs in that its constitutive association with mIg causes it to be phosphorylated and to bind SHP without a requirement for cross-linking to mIg. Thus, in contrast to FcγRIIB, the purpose of ligating CD22 may be to remove it from the vicinity of mIg in order to release the cell from the negative regulatory effects of SHP.

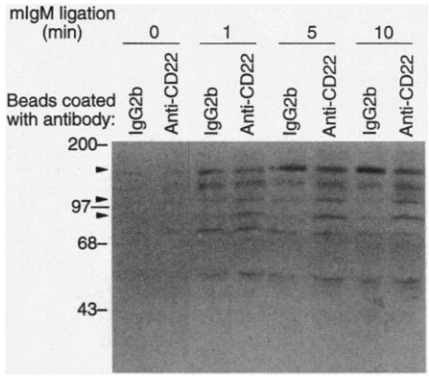

Fig. 4. Effect of immobilization of CD22 on tyrosine phosphorylation of in B lymphocytes stimulated by ligating mIgM. Replicate samples of 1 × 10^6 purified, high-density, tonsillar B cells (*18*) were preincubated with beads bearing IgG2b of irrelevant specificity or with beads bearing antibody to CD22. The cells were then stimulated with DA4.4 antibody to IgM (5 μg/ml) and Il-4 (200 U/ml) for timed intervals. Reactions were terminated by the addition of NP-40 lysis buffer. The lysates were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose. The blots were probed with ^{125}I-4G10 antibody to phosphotyrosine. Molecular masses are given at left in kilodaltons.

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12. The antibodies used in this study were B3 antibody to human CD22 (Coulter, Hileah, FL), CY34.1.2 antibody to murine CD22 (ATCC), B4 antibody to human CD19 (Coulter), control mouse IgG2b (Southern Biotechnology Associates, Birmingham, AL), RG7/9.1 (mouse IgG2b, antibody to rat κ light chain, from T. Springer, Harvard Medical School, Boston, MA), goat F(ab′)$_2$ antibody to human IgM (Tago, Burlingame, CA), 4G10 antibody to phosphotyrosine (UBI, Lake Placid, NY), rabbit polyclonal antisera raised against a GST-SHP fusion protein (UBI), and peroxidase-coupled mouse antibody to rabbit IgG (Jackson Immunoresearch).
13. Lymphocytes were lysed with ice-cold lysis buffer containing 1% NP-40, 50 mM tris (pH 7.4), 10 mM EDTA, 80 mM KCl, 10 mM sodium molybdate, 1 mM sodium orthovanadate, 5 mM iodoacetamide, 10 μM leupeptin, antipain (2 μg/ml), 1 μM pepstatin A, chymostatin (6 μg/ml), aprotinin (1 μg/ml), and 4-(2-aminoethyl)-benzenesulfonyl fluoride (1 μg/ml)(Calbiochem). The soluble portion of lysates was incubated with an excess of precipitating antibody followed by protein A–trisacryl beads (Pierce). Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose membranes were probed with 4G10 (0.1 mg/ml) that had been labeled with ^{125}I, and dried membranes were exposed to XOMAT film (Kodak) at −80°C with the use of an intensifying screen. Membranes were reprobed with a 1:100 dilution of rabbit antibody to SHP and then incubated with peroxidase-conjugated mouse antibody to rabbit IgG. Blots were washed and visualized with the enhanced chemiluminescence (ECL) detection system (Amersham).
14. Resting murine splenic B cells [J. Lin and L. B. Justement, *J. Immunol.* **149**, 1548 (1992)] were permeabilized [C. M. Pleiman, W. M. Hertz, J. C. Cambier, *Science* **263**, 1609 (1994)] and incubated with phosphotyrosyl peptides corresponding to the sequence of murine CD22. Murine nonphosphorylated peptides and human phosphotyrosyl peptides corresponding to tyrosine-containing regions of cytoplasmic CD22 were synthesized with the use of fluorenyl methoxycarbonyl chemistry on an Applied Biosystems 432 A Synthesizer. Murine phosphotyrosyl peptides were purchased from Quality Controlled Biochemicals (Hopkinton, MA). After deblocking, the peptides were purified by reversed-phase high-pressure liquid chromatography. Sequences and tyrosine phophorylation were confirmed by mass spectrometry. The cells were brought to 37°C and were incubated with 100 μM sodium orthovanadate and 0.2 mM H_2O_2 for 10 min. Cells were pelleted; resuspended in lysis buffer containing 10 mM tris-HCl (pH 7.3), 150 mM NaCl, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), aprotinin (1 μg/ml), α–anti-trypsin (1 μg/ml), and 1% digitonin; and precleared with RG7/9.1 coupled to Sepharose, followed by immunoprecipitation of CD22 with CY34.1.2 antibody to CD22 coupled to Sepharose. Immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit antibody to SHP followed by ECL.
15. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, any amino acid; and Y, Tyr.
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17. A Bluescript plasmid encoding the full-length complementary DNA (cDNA) for SHP was used as a template for the generation by polymerase chain reaction of a cDNA fragment encoding full-length SHP. This was directionally cloned into pGEX and GST fusion proteins were expressed and purified.
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19. Antibodies were covalently coupled with carbodiimide linkage to carboxylated latex microparticles 2 μm in diameter (Polysciences, Warrington, PA) according to the manufacturer's instructions.
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