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Immunology

Bruton tyrosine kinase is tyrosine phosphorylated and activated in pre-B lymphocytes and receptor-ligated B cells

(X chromosome-linked agammaglobulinemia/pre-antigen receptor/B-cell signaling)

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ABSTRACT **The gene encoding Bruton tyrosine kinase (Btk) is known to be mutated in human X chromosome-linked agammaglobulinemia and in the Xid mouse. This kinase was examined in B lymphocytes before and after antigen receptor ligation and also in pre-B cells. Btk was found to be catalytically activated and tyrosine phosphorylated in response to anti-IgM stimulation in B cells. This kinase is also constitutively phosphorylated on tyrosine residues in pre-B cells. These findings point to a functional role for Btk in pre-antigen and antigen receptor signaling during B-cell development and provide a biochemical explanation for the X-linked genetic syndromes already linked to this kinase.**

Bruton tyrosine kinase (Btk) is a member of a small family of tyrosine kinases that share certain structural features, but whose biological roles are poorly understood. These kinases, which include Itk/Tsk in T lymphocytes (1, 2), TEC I and TEC II in liver cells as well as hematopoietic cells (3, 4), and the DSrc28 kinase in *Drosophila* (5), have conserved kinase, Src homology 2 (SH2), and SH3 domains but lack N-terminal myristoylation signals and C-terminal negative regulatory tyrosine residues. Human and mouse genetic disorders in which Btk is defective indicate that this kinase plays an important role in B-cell differentiation. The specific function of this kinase in B-lineage cells remains to be elucidated. Mutations in Btk that compromise catalytic activity result in the failure of human pre-B lymphocytes to mature efficiently into surface IgM-positive B cells (6, 7). Other mutations, outside the kinase domain, some of which may be predicted to compromise the function of Btk, lead to a variable degree of impairment of B-cell generation. Collectively, these mutations constitute the clinical spectrum of X chromosome-linked agammaglobulinemia (8, 9). A point mutation in the N-terminal pleckstrin homology domain of this kinase has been detected in the Xid mouse (10, 11). In these mice the pre-B to B-cell transition is minimally impaired, but B cells fail to respond to polyvalent antigens and do not proliferate in response to soluble anti-IgM antibodies *in vitro*. Btk has been reported to be localized to the cytoplasm, but a biochemical role for this kinase during B-cell differentiation has thus far been lacking.

During B-cell differentiation pre-B cells that make in-frame rearrangements at the heavy chain locus presumably receive a survival signal from the pre-B receptor and are positively selected. The pre-B receptor is made up of membrane immunoglobulin heavy chains, surrogate light chains (12–16), and other antigen receptor-associated proteins (17). Inactivation by homologous recombination of μm or of the surrogate light chain encoding $\lambda 5$ gene (18) leads to a block in differentiation at a late pre-B stage, generating a phenotype in mice that resembles that seen in some patients with X-linked agammaglobulinemia. However, to date, there has been no evidence to biochemically link Btk to signal transduction via the pre-B or B-cell receptor.

In this report we present evidence for the catalytic activation of Btk following receptor ligation in B cells. Crosslinking of membrane immunoglobulin also leads to the phosphorylation of Btk on tyrosine residues in B cells; in pre-B cells Btk is constitutively tyrosine phosphorylated. These results provide a biochemical link between signal transduction via the B-cell receptor and Btk. The tyrosine phosphorylation of Btk in pre-B cells supports models for constitutive signaling via the pre-B receptor. These findings suggest that the defect in X-linked agammaglobulinemia represents the inability to generate a signal for the positive selection of B-lineage cells that have productively rearranged their heavy chain loci.

MATERIALS AND METHODS

Cell Lines. The source of WEHI 231, a surface IgM-expressing immature B-cell line, and the derivation of the NFS 70.15 pre-B-cell line have been described earlier (19).

Anti-Btk Antibodies. Anti-Btk antibodies were raised in rabbits using a glutathione *S*-transferase (GST) Btk fusion as described by Tsukada *et al.* (7) followed by depletion over a GST-Sepharose immunoadsorbent and affinity purification using GST-Btk-Sepharose.

Anti-Phosphotyrosine Immunoprecipitation and Anti-Btk Immunoblot Analysis. Anti-IgM crosslinking was performed and lysates from 5×10^7 cells (in 1% Nonidet P-40) were prepared as described (19). Four-fifths of each lysate was used for the anti-phosphotyrosine immunoprecipitation step, using 50 μl of a 50% slurry of a monoclonal anti-phosphotyrosine antibody coupled to agarose (PY 20, Zymed). The remaining fifth of each lysate was immunoprecipitated with anti-Btk. Immunoprecipitates were washed four times with 0.2% Nonidet P-40 in 10 mM Tris, pH 8.0/120 mM NaCl/0.4 mM EDTA, separated on a 10% polyacrylamide/SDS gel, and transferred to an Immobilon-P membrane, and Btk was revealed by immunoblotting, using the ECL system (Amersham). In separate experiments, lysates were immunoprecipitated with anti-Btk as described above and an anti-phosphotyrosine immunoblot was performed as described (19).

Assay for Catalytic Activation of Btk. WEHI 231 B cells (3×10^6) were used for each time point of the *in vitro* kinase assay. Cells (1.3×10^7) were stimulated with 2.5 μg of $F(ab')_2$ anti-IgM (Zymed) in 100 μl of serum-free RPMI medium at 37°C. At various time points lysates were made as described above and precleared using rabbit IgG and protein A-Sepharose. A portion of each lysate representing 3×10^6 cells was immunoprecipitated with anti-Btk, washed four times with the 0.2% Nonidet P-40 buffer described in the previous

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Abbreviations: Btk, Bruton tyrosine kinase; SH, Src homology.

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section, and then washed once with 20 mM Pipes (pH 7.0) containing 20 mM $MnCl_2$. The beads were taken up in 20 μl of the above buffer and mixed with 2.5 μg of acid-denatured rabbit muscle enolase and 10 μCi of [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). After 5 min at 30°C, the reaction was terminated and samples were separated on a 9% polyacrylamide/SDS gel. After fixation, the gel was treated with 1 M KOH at 60°C for 90 min, refixed, dried, and exposed for autoradiography. A separate portion of each lysate representing 10^7 cells was analyzed for Btk using the above immunoprecipitation/immunoblot approach.

RESULTS

WEHI 231, a surface IgM-expressing murine B-cell line, has been extensively used in studies of signal transduction mediated by the B-lymphocyte antigen receptor (20, 21). We initially examined whether Btk was phosphorylated on tyrosine residues in response to the crosslinking of membrane IgM in this cell line, and we also studied a pre-B-cell line, NFS 70.15 (19), that had been derived from a pro-B-cell line immortalized by the nuclear v-*cbl* protein (presumed to be a transcription factor). It was particularly important to study such a line, since Abelson virus-derived pre-B lines express an active transforming tyrosine kinase and are unsuitable for studies of signal transduction. NFS 70.15 expresses intracellular pre-B receptor complexes, containing μm, surrogate light chains, the immunoglobulin α/β heterodimer, and associated Src family kinases. In previous studies (19, 22) a number of specific SH2 domain binding proteins were detected in activated B-cell lines (but not in nonstimulated cells) including WEHI 231 (19). In the NFS 70.15 pre-B-cell line, however, some of these above tyrosine phosphorylated proteins were also detected in the absence of any extraneous stimulation (19), indirectly supporting models invoking the constitutive ligand-independent activation of the pre-B receptor (13, 16, 23).

Btk Is Tyrosine Phosphorylated in Response to Receptor Ligation in B Cells and Constitutively in Pre-B Cells. We wished to determine whether Btk was tyrosine phosphorylated in response to anti-IgM stimulation in B cells and whether it was constitutively tyrosine phosphorylated in the NFS 70.15 pre-B-cell line. Phosphotyrosine-containing proteins were affinity purified from pre-B- and B-cell lysates on an anti-phosphotyrosine monoclonal antibody matrix and Btk was revealed using an affinity-purified anti-Btk antibody (Fig. 1, lanes 1–3). A portion of each lysate was also immunoprecipitated with anti-Btk antibodies to verify that Btk was actually present in equivalent amounts (lanes 4–6). Btk was tyrosine phosphorylated after antigen receptor crosslinking in WEHI 231 B cells (lane 3) but not in nonstimulated B cells (lane 2); this enzyme is constitutively phosphorylated in NFS 70.15 pre-B cells (lane 1). Immunoprecipitation using anti-Btk followed by an anti-phosphotyrosine immunoblot assay (data not shown) also detects phosphotyrosinylated Btk. This latter approach does not reveal other phosphorylated molecules that might have been postulated to be tightly associated with Btk. In theory, a phosphoprotein noncovalently associated with Btk could potentially have mediated the latter's binding to the anti-phosphotyrosine matrix used in experiments such as the one depicted above.

Catalytic Activation of Btk Following Receptor Ligation in B Cells. Ligation of the antigen receptor in WEHI 231 cells leads to an increase in the catalytic activity of Btk as assessed both by autophosphorylation and the phosphorylation of an exogenous substrate, enolase. Previous studies (7) have demonstrated that Btk is autophosphorylated on tyrosine residues. As seen in Fig. 2*A*, kinase activity peaks around 3–5 min and appears to decline subsequently. We confirmed that the amount of Btk in lysates at time points at which

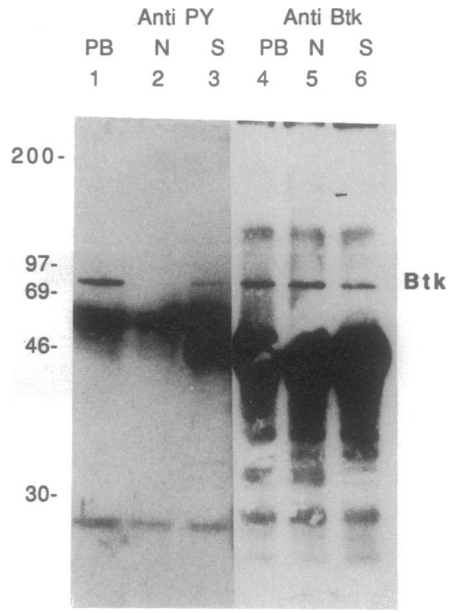

FIG. 1. Btk is phosphorylated on tyrosine residues in response to antigen receptor ligation in WEHI 231 B cells and constitutively in NFS 70.15 pre-B cells. Btk was revealed by an anti-phosphotyrosine immunoblot. Lanes 1 and 4, lysates from NFS 70.15 cells; lanes 2 and 5, lysates from nonstimulated WEHI 231 cells; lanes 3 and 6, lysates from anti-IgM stimulated WEHI 231 cells. In lanes 1–3, samples were immunoprecipitated with an anti-phosphotyrosine monoclonal antibody. In lanes 4–6, lysates were immunoprecipitated with anti-Btk. Anti PY, anti-phosphotyrosine; PB, pre-B; N, nonstimulated; S, stimulated (by receptor ligation). Molecular weights are indicated as $M_r \times 10^{-3}$.

catalytic activity peaked was either equivalent or less than that at earlier time points (Fig. 2*B*). The decline of catalytic activity after it peaks appears in part to be an artefact of the decreased availability of Btk in detergent extracts after receptor ligation (as seen in Fig. 2*B*). In fact, the peak of catalytic activity in Fig. 2*A* at 3 min is seen at a point when the Btk content of the same lysate (as seen in Fig. 2*B*) is less than at earlier time points. Fig. 2*C* shows results from an experiment similar to that in Fig. 2*A*. In this exposure the phosphorylation of enolase, between 3 and 5 min after the crosslinking stimulus, is more readily visualized.

DISCUSSION

The above findings place Btk along the pathways of pre-antigen receptor and antigen receptor-mediated signaling in B-lineage cells. Crosslinking of membrane immunoglobulin in B cells leads to the tyrosine phosphorylation and catalytic activation of Btk, indicating that B-cell receptor-mediated signal transduction targets this tyrosine kinase. In pre-B cells, Btk is constitutively tyrosine phosphorylated. This finding supports the view that assembly itself of the pre-B receptor might provide the signal for the activation of this receptor and the consequent catalytic activation of Btk. Pre-antigen receptors (the pre-B receptor in the B lineage and the pre-T receptor comprising the T-cell receptor β chain and a surrogate α chain in the T lineage; ref. 24) are unique in that they are not designed to monitor the external milieu of the cells in which they are expressed but, on the contrary, may have evolved to monitor productive rearrangement of immunoglobulin heavy chain and T-cell receptor β chain genes. The defect in X-linked agammaglobulinemia presumably compromises the pre-B receptor-mediated survival of B-lineage cells that have productively rearranged their heavy chain loci.

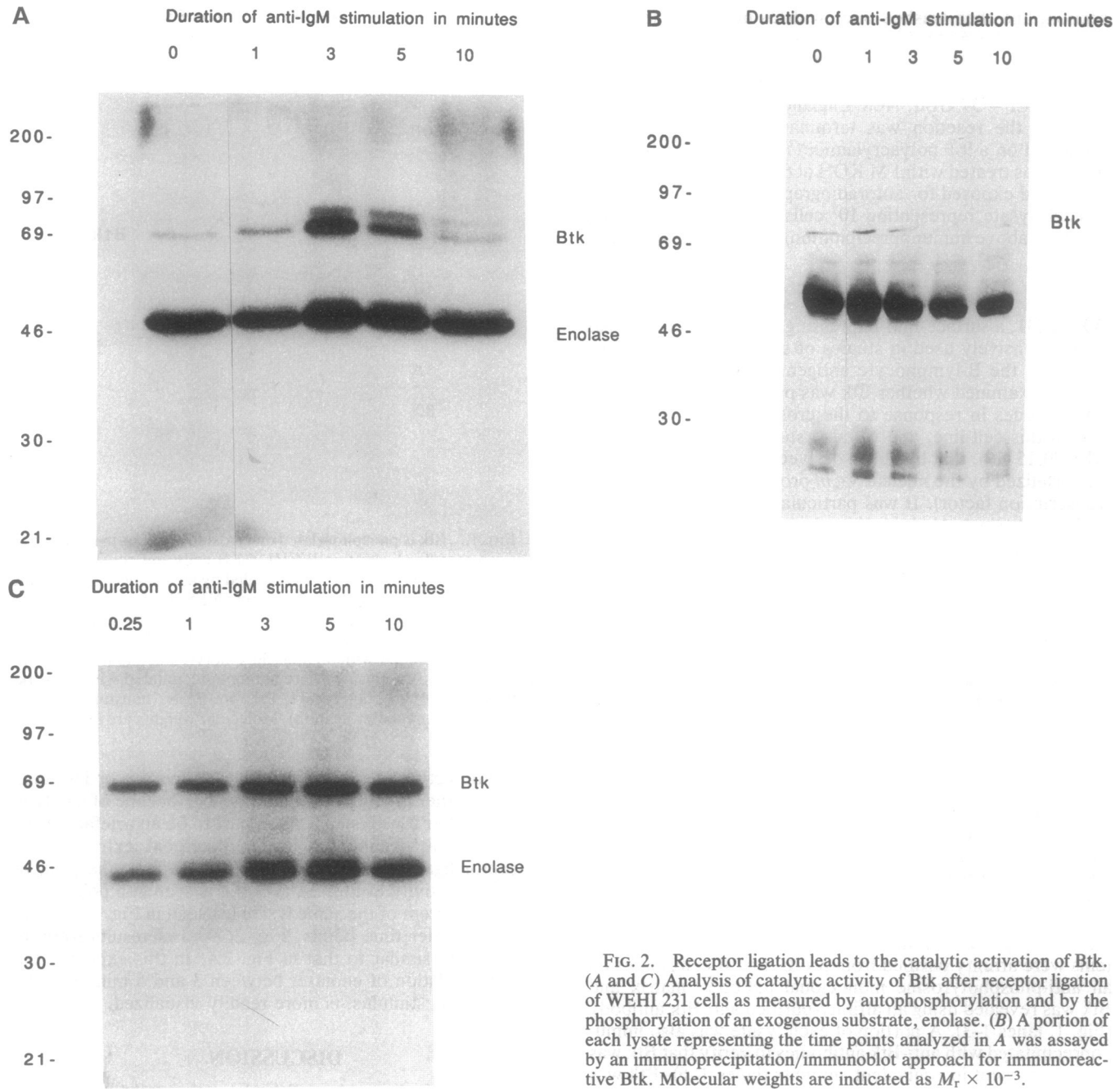

FIG. 2. Receptor ligation leads to the catalytic activation of Btk. (*A* and *C*) Analysis of catalytic activity of Btk after receptor ligation of WEHI 231 cells as measured by autophosphorylation and by the phosphorylation of an exogenous substrate, enolase. (*B*) A portion of each lysate representing the time points analyzed in *A* was assayed by an immunoprecipitation/immunoblot approach for immunoreactive Btk. Molecular weights are indicated as $M_r \times 10^{-3}$.

The B-cell receptor is made up of membrane immunoglobulin heavy chains, associated light chains, the immunoglobulin α/β heterodimer, associated Src family kinases, and the tandem SH2 domain containing Syk kinase (17, 25). Src family kinases known to be associated with this receptor include Blk, Lyn, Fyn, and Lck (21, 26, 27). Tyrosine phosphorylation is known to be involved in the process of signal transduction (20) and catalytic activation of Src family kinases and Syk has been demonstrated in B cells activated with anti-IgM. Src family kinases are presumed to associate with the ARH1 motifs in the cytoplasmic tails of Igα and Igβ. The mechanism by which Syk is associated with this receptor remains unclear (28), although it might well be recruited (29, 30) to the ARH1 motifs in activated B cells in a manner analagous to the presumed recruitment of ZAP 70 by the ARH1 motifs in the ς chain of the T-cell receptor. On the basis of the above evidence it is likely that Btk physically interacts with the B-cell receptor and the pre-B receptor. Preliminary immunoprecipitation/reimmunoprecipitation experiments support such a conclusion (Y.A. and S.P., unpublished observations). The structural basis for the interaction of these receptors with Btk is an issue of considerable interest. The catalytic activation of Btk either is presumably a consequence of a tyrosine phosphorylation-dependent conformational change in Btk or reflects the association of phosphorylated Btk with another protein possibly through an SH2-type interaction. These mechanistic issues remain to be explored.

What is the nature of the signal generated by Btk? In the Xid mouse the point mutation in the pleckstrin homology domain apparently does not compromise the function of Btk in positive selection at the pre-B stage. Crosslinking of membrane immunoglobulin in splenic B cells of these mice with soluble anti-IgM does not affect early events in B-cell signaling (31) but fails to deliver a proliferative signal. Since proliferation can be induced by a crosslinking stimulus of greater magnitude, it is possible that Btk mediates similar function at the pre-B and B stages but that a quantitatively smaller signal suffices for pre-B rescue. Alternatively, the

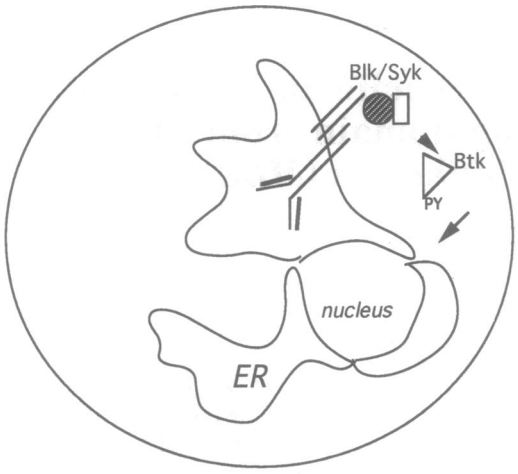

FIG. 3. Model for constitutive/ligand-independent pre-B receptor-mediated signaling involving Btk. ER, endoplasmic reticulum; PY, phosphotyrosine. Depicted is a pre-B receptor complex consisting of surrogate light chains associated with μm, which in turn is associated with the immunoglobulin α/β heterodimer (only one associated heterodimer is depicted). Blk is depicted as a shaded circle and Syk is depicted as a rectangle. The mechanism of association of these proteins with the receptor is unclear; Blk (and other Src family kinases) is presumed to associate with the cytoplasmic tails of the α/β heterodimer, and it is likely that Syk is recruited to the receptor after crosslinking. Evidence exists for the formation of a complex between Blk and Syk (Y.A., R. Stillwell, and S.P., unpublished observations) and this association is depicted. Btk is tyrosine phosphorylated and catalytically activated and provides a signal that mediates pre-B rescue and allelic exclusion.

pleckstrin homology domain may be responsible for a B-stage-specific interaction of Btk with a target. It has been suggested that pre-antigen receptors might mediate allelic exclusion by the induction of lymphocyte entry into S phase (32) and the consequent phosphorylation and degradation of RAG-2 (33). It is conceivable that Btk is responsible for a nuclear event critical to the entry of pre-B cells and of resting B cells into S phase (Fig. 3). Allelic exclusion in T cells appears to be mediated by a signal generated from the T-cell receptor β-gp33 complex (24), which presumably also includes $p56^{lck}$ (34). Although Blk is specific to B-lineage cells (35) it is unclear which Src family kinase, if any, plays a critical role in pre-B signaling for rescue and allelic exclusion. Given the many similarities between the early events in B- and T-lymphocyte development, it is likely that Itk/Tsk, the T-lineage homolog of Btk, might play an important role in the first positive selection step in T-cell development.

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1. Siliciano, J. D., Morrow, T. A. & Desiderio, S. V. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11194–11198.
2. Heyeck, S. D. & Berg, L. J. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 669–673.
3. Mano, H., Ishikawa, F., Nishida, J., Hirai, H. & Takaku, F. (1990) *Oncogene* **5,** 1781–1786.
4. Mano, H., Mano, K., Tang, B., Koehler, M., Yi, T., Gilbert, D. J., Jenkins, N. A., Copeland, N. G. & Ihle, J. N. (1993) *Oncogene* **8,** 417–424.
5. Gregory, R. J., Kammermeyer, K. L., Vincent, W. S., III, & Wadsworth, S. G. (1990) *Mol. Cell. Biol.* **7,** 2117–2127.
6. Vetrie, D., Vorechovsky, I., Sideras, P., Holland, J., Davies, A., Flintner, F., Hammarstrom, L., Kinnon, C., Levinsky, R., Bobrow, M., Smith, C. I. E. & Bentley, D. R. (1993) *Nature (London)* **361,** 226–233.
7. Tsukada, S., Saffran, D. C., Rawlings, D. J., Parolini, O., Allen, R. C., Klisak, I., Sparkes, R. S., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J. W., Saper, M. D., Conley, M. E. & Witte, O. N. (1993) *Cell* **72,** 279–290.
8. Saffran, D. C., Parolini, O., Fitch-Hilgenberg, M. E., Rawlings, D. J., Afar, D. E. H., Witte, O. N. & Conley, M. E. (1994) *N. Engl. J. Med.* **330,** 1488–1491.
9. Buckley, R. H. (1994) *N. Engl. J. Med.* **330,** 1526–1528.
10. Thomas, J. D., Sideras, P., Smith, C. I. E., Vorechovsky, I., Chapman, V. & Paul, W. E. (1993) *Science* **261,** 355–358.
11. Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., Jenkins, N. A. & Witte, O. N. (1993) *Science* **261,** 358–361.
12. Pillai, S. & Baltimore, D. (1987) *Nature (London)* **329,** 172–174.
13. Pillai, S. & Baltimore, D. (1988) *Curr. Top. Microbiol. Immunol.* **137,** 136–139.
14. Karasuyama, H., Kudo, A. & Melchers, F. (1990) *J. Exp. Med.* **172,** 969–972.
15. Tsubata, T. & Reth, M. (1990) *J. Exp. Med.* **172,** 973–975.
16. Cherayil, B. J. & Pillai, S. (1991) *J. Exp. Med.* **1734,** 111–116.
17. Reth, M. (1992) *Annu. Rev. Immunol.* **10,** 97–121.
18. Ehlich, A., Schaal, S., Gu, H., Kitamura, D., Muller, W. & Rajewsky, K. (1993) *Cell* **72,** 695–704.
19. Aoki, Y., Isselbacher, K. J., Cherayil, B. J. & Pillai, S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4204–4208.
20. Gold, M. R., Law, D. A. & DeFranco, A. L. (1990) *Nature (London)* **345,** 810–813.
21. Campbell, M. A. & Sefton, B. M. (1992) *Mol. Cell. Biol.* **12,** 2315–2321.
22. Malek, S. N. & Desiderio, S. V. (1993) *J. Biol. Chem.* **268,** 22557–22665.
23. Pillai, S. (1991) *Int. Rev. Cytol.* **130,** 1–34.
24. Groettrup, M., Ungeweiss, K., Azogul, O., Palacios, R., Owen, M. J., Hayday, A. C. & von Boehmer, H. (1993) *Cell* **75,** 283–294.
25. Cambier, J. C., Bedzyk, W., Campbell, K., Chien, N., Friedrich, J., Harwood, A., Jensen, W., Pleiman, C. & Clark, M. C. (1993) *Immunol. Rev.* **132,** 85–106.
26. Burkhardt, A. L., Brunswick, M., Bolen, J. R. & Mond, J. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7410–7414.
27. Yamanashi, Y., Kakiuchi, T., Mizuguchi, J., Yamamoto, T. & Toyoshima, K. (1991) *Science* **251,** 192–194.
28. Hutchcroft, J. E., Harrison, M. L. & Geahlen, R. L. (1992) *J. Biol. Chem.* **267,** 8613–8619.
29. Law, D. A., Chan, V. W. F., Datta, S. K. & DeFranco, A. L. (1993) *Curr. Biol.* **3,** 645–657.
30. Law, C-L., Sidorenko, S. P., Chandran, K. A., Draves, K. E., Chan, A. C., Weiss, A., Edelhoff, S., Disteche, C. M. & Clark, E. A. (1994) *J. Biol. Chem.* **269,** 12310–12319.
31. Lindsberg, M-L., Brunswick, M., Yamada, H., Lees, A., Inman, J., June, C. H. & Mond, J. J. (1991) *J. Immunol.* **147,** 3774–3779.
32. Dudley, E. C., Petrie, H. P., Shah, L. M., Owen, M. J. & Hayday, A. C. (1994) *Immunity* **1,** 83–93.
33. Lin, W. C. & Desiderio, S. V. (1993) *Science* **260,** 953–959.
34. Anderson, S. J., Levin, S. D. & Perlmutter, R. M. (1993) *Nature (London)* **365,** 552–554.
35. Dymecki, S. M., Niederhuber, J. E. & Desiderio, S. V. (1990) *Science* **247,** 332–336.