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Biochemistry

Membranes from T and B lymphocytes have different patterns of tyrosine phosphorylation

(tyrosine-specific kinase)

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ABSTRACT **Membrane fractions isolated from mouse and rat spleen expressed substantial tyrosine-specific protein kinase activity. Phosphotyrosine (*P*-Tyr) accumulation in endogenous membrane substrates was stimulated by vanadate or nonionic detergents. When *in vitro* phosphorylation was carried out at 0°C in the presence of 1 mM Mn^{2+} and Triton X-100, *P*-Tyr constituted up to 40–50% of the total phospho amino acid. Polyacrylamide gel electrophoresis showed that membranes from mixed lymphocyte populations have four major *P*-Tyr-containing proteins. Whereas nonionic detergents were potent stimuli for *P*-Tyr accumulation in all four substrates, tyrosine phosphorylation of two of these (p61 and p55) was markedly dependent on vanadate. These two substrates were present in membranes from surface Ig-bearing splenic lymphocytes purified by affinity chromatography and Raji, a human B-lymphoblastoid cell line. *P*-Tyr accumulation in the two other substrates observed in splenocyte membranes (p64 and p58) was much less dependent on vanadate. p64 and p58 were phosphorylated in membranes from mouse thymocytes and human and mouse T-lymphoma cell lines, while p61 and p55 were not. Thus it appears that in both murine and human lymphocytes, p64 and p58 served as T-cell-specific substrates, while p61 and p55 were specifically associated with B lymphocytes. Moreover, these distinct *P*-Tyr substrate patterns were conserved in some neoplastic cell lines derived from B and T cells.**

Studies of cellular transformation by oncogenic viruses revealed a class of protein kinases that phosphorylate tyrosine residues (1–5). The transforming gene products of certain RNA tumor viruses have intrinsic tyrosine-specific kinase activity and serve as substrates for tyrosine-specific kinases (5). The supposition that this activity was confined to viral products was quickly dispelled by two findings. First, the transforming gene of Rous sarcoma virus was found to be the homolog of a normal cellular gene (6, 7). Subsequently, more than 15 viral transforming genes have been found to be derived in part from cellular genetic information; at least 5 have tyrosine-specific kinase activity (5, 7). Second, the membrane receptor for epidermal growth factor was shown to have kinase activity with tyrosine specificity (8–10). The membrane receptors for platelet-derived growth factor (11), insulin (12), and somatomedin C (13) have also been shown to possess ligand-activated tyrosine-specific kinase activity. As is the case with the viral products, these kinases are also substrates for tyrosine phosphorylation. The association with oncogenic transformation, growth factor receptors, and increased activity early in embryogenesis (14) has linked the tyrosine kinases to the regulation of growth and development.

Workers in our laboratory have studied phosphotyrosine (*P*-Tyr) accumulation in isolated membranes using dimethyl sulfoxide (15–17) and vanadate (18) as chemically defined stimuli. Membranes from Raji, a human Burkitt lymphoma-derived B-lymphoblastoid cell line, contain two substrates that were selectively phosphorylated on tyrosine residues in the presence of vanadate (18). The effective concentration of vanadate, a known inhibitor of Na^+/K^+ ATPase and various phosphatase activities, was within the physiologic range found in blood and tissue (19–22). To determine whether this activity was related to the transformed state, we examined tyrosine phosphorylation in membranes from normal lymphoid tissue. Tyrosine kinase activity was present in normal spleen, a finding substantiated by Swarup *et al.* (23). Vanadate stimulated splenic membrane *P*-Tyr accumulation. The characteristics of the reaction and the size of the two major vanadate-sensitive substrates were similar to that of Raji membranes. Casnellie *et al.* (24) had shown that a mouse T-cell lymphoma line, LSTRA, contained a tyrosine substrate that had a slightly different molecular weight than we had observed in B lymphocytes. Examination of membranes from thymocytes, splenocytes, purified splenic B cells, and lymphoid cell lines demonstrated that the tyrosine phosphoprotein pattern of T and B lymphocytes was distinct. The characteristics of *in vitro* tyrosine phosphorylation may provide a new means to distinguish lymphocyte populations.

METHODS

Cell and Membrane Preparation. Rat spleens were homogenized in 0.25 M sucrose/10 mM PO_4/1 mM EDTA, with a Brinkman Polytron apparatus (setting 7.5; 30 sec). After centrifugation at 30,000 × *g* for 7.5 min, the supernate and loose material above the compact pellet were centrifuged at 105,000 × *g* for 1 hr. Cell suspensions and cultured cells were washed with 0.15 M NaCl and homogenized in 10 mM PO_4, pH 7.4/1 mM EDTA (setting 7.5, 10 sec, two times). An equal volume of buffer containing 0.5 M sucrose was added. After centrifugation at 1000 rpm for 5 min, the supernate was centrifuged at 105,000 × *g* (1 hr). Membrane pellets were suspended in 20 mM Pipes (pH 7.0) and the protein concentration was determined (25).

Raji cells were grown in suspension culture (10^6 cells per ml) as described (18). The mouse T-cell lines BALB-D-1 (Bd-1) (26), EL-4 (27), and RBL-5 (28) were grown in RPMI 1640 medium supplemented to 10% with fetal calf serum to a density of 10^6 cells per ml as was HSB, a human T-cell line (29). Minced fragments of thymi or spleens from several BALB/c male mice, 5–8 weeks old, were dissociated into single cell suspensions mechanically by gentle trituration at 4°C with a serological pipet (30). Erythrocytes contaminating the splenocyte suspensions were removed by hypotonic lysis in water, by ammonium chloride lysis, or by differential density gradient sedimentation on Ficoll-Hypaque gradients.

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Abbreviations: *P*-Tyr, phosphotyrosine; sIg, surface immunoglobulin.

Approximately 75–100 × 10^6 cells with >95% viability (trypan blue-excluding) were routinely obtained from each thymus or spleen by this method. Cells bearing Thy-1.2 or surface immunoglobulin (sIg) were simultaneously enumerated by direct immunofluorescence microscopy using fluoresceinated monoclonal anti-Thy-1.2 [HO-13-4] (31) and rhodamine-conjugated goat $F(ab')_2$ anti-mouse IgG (32). Splenic B lymphocytes (sIg^+) were purified by specific adsorption to, and elution from, an Affi-Gel anti-mouse immunoglobulin affinity column (Bio-Rad).

Phosphorylation and Electrophoresis. Membrane protein (50 μg) was preincubated for 10 min at 0°C with H_2O, vanadate (Na_3VO_4) or Triton X-100 in 50 μl containing 50 mM Pipes (pH 7.0) and 1 mM $MnCl_2$ or 30 mM $MgCl_2$. A 60-sec phosphorylation reaction was begun by adding 1 μM ATP, (5 μCi [γ-^{32}P]ATP; 1 Ci = 37 GBq). Samples were subjected to $NaDodSO_4$/8% or 10% polyacrylamide slab gel electrophoresis followed by autoradiography as described (16). To obtain a relative indication of *P*-Tyr-containing substrates, selected wet gels were treated for 2 hr with 1 M KOH (55°C) as described by Cooper and Hunter (33). Molecular weights were estimated by using low molecular weight standards from Bio-Rad.

Phospho Amino Acid Analysis. After phosphorylating 1 mg of membrane protein, the protein was hydrolyzed and phospho amino analysis was carried out by a two-dimensional technique described by Hunter and Sefton (2). Individual bands were analyzed after identifying and cutting out p61 or p58 from a Coomassie blue-stained wet gel. Subsequent autoradiography of the gel from which each band was cut confirmed the accuracy of each band excision. While the regions of p64, p61, p58, and p55 are each minimally stained by Coomassie blue, their locations can be estimated from more heavily stained bands that are reproducibly adjacent. Protein was electrophoretically eluted from the gel slice into a dialysis bag, and bovine serum albumin (1 mg) was added. After trichloroacetic acid precipitation, washing, and hydrolysis (6 M HCl, 110°C, 3 hr), phospho amino acid was separated and quantified by liquid scintillation counting as described (16, 18).

RESULTS

Membrane preparations from rat spleen were assayed in the presence of 30 mM Mg^{2+}. Vanadate (1–30 μM) stimulated the phosphorylation of two splenic membrane proteins (Fig. 1). Their molecular weights, 61,000 and 55,000 (p61 and p55), were the same as those found in Raji cell membranes. *P*-Tyr is alkali resistant and remains protein associated after treatment of gels with KOH; *P*-Ser and *P*-Thr are alkali labile. When gels from similar experiments were treated with KOH, almost no alkali-resistant phosphate was present in the absence of vanadate. The phosphate incorporated into p61 and p55 induced by vanadate was KOH resistant (data not shown). Vanadate-stimulated p61 and p55 phosphorylation was also seen with 1 mM Mn^{2+} but not with 5 mM Ca^{2+}.

Phospho amino acid analysis after *in vitro* phosphorylation of splenic membranes (Table 1) demonstrated that vanadate increased *P*-Tyr accumulation 3- to 4-fold in the presence of 1 mM Mn^{2+}. Additional experiments with vanadate using membranes from mouse spleen, mouse splenocytes, and Raji cells, did not reveal consistent alterations in *P*-Ser or *P*-Thr, but *P*-Tyr increased 2.5- to 6-fold. We have previously established that under these conditions (1 min, 0°C) vanadate does not alter ATP availability (18).

Attempts to solubilize the kinase and/or substrate revealed another biochemical characteristic of the *in vitro* lymphocyte tyrosine phosphorylation. When added to the reaction mixture, increasing concentrations of Triton X-100 promoted phosphorylation of Raji membrane p61 and p55 (Fig. 2). Ultracentrifugation of Triton-treated membranes showed that the tyrosine-specific kinase(s) and phosphoproteins were not solubilized but remained in the pellet. Similarly, Nonidet P-40 stimulated tyrosine-specific phosphorylation but failed to solubilize the substrates. Concentrations of Triton >0.1% increased p61 and p55 phosphorylation such that no further phosphorylation could be discerned when vanadate was added (data not shown). Phospho amino acid analysis of splenic membranes phosphorylated in the presence of 0.6% Triton X-100 and 1 mM Mn^{2+} showed that *P*-Tyr content was 40–50% of the total phospho amino acid.

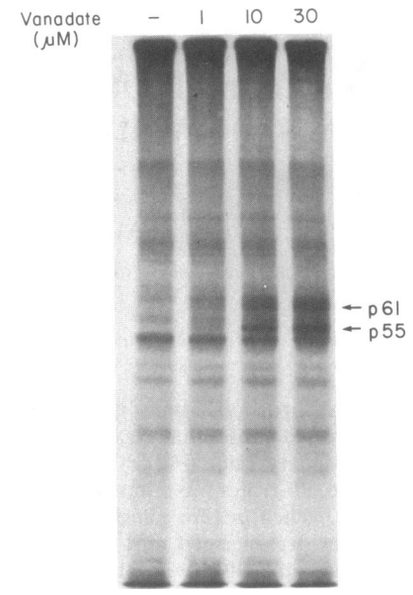

FIG. 1. *In vitro* phosphorylation of endogenous rat splenic membrane substrates. Membrane fractions (50 μg) were incubated for 10 min at 0°C with 30 mM Mg^{2+} and the indicated concentrations of test substances, in this case vanadate. A 60-sec reaction at 0°C was begun by adding 1 μM ATP (5 μCi [γ-^{32}P]ATP). After $NaDodSO_4$/8% PAGE, gels were autoradiographed.

We next sought to determine whether or not T and B lymphocytes have distinct tyrosine phosphoproteins. Single cell suspensions of mouse thymocytes and splenocytes were prepared and the relative proportion of T and B cells was estimated by direct immunofluorescence. Thy-1.2 was expressed by 98% of the thymocytes. Splenocytes contained two major populations: 40% expressed sIg (B cells) and 45–50% were Thy-1.2 positive (T cells). Membranes were phosphorylated at 0°C with or without 30 μM vanadate. Duplicate samples were electrophoresed on the same gel. The gel was cut and autoradiographed with or without prior KOH treatment (Fig. 3). Thymocytes contained two major phospho-

Table 1. Phospho amino acid content of rat splenic membranes

	P-Ser		*P*-Thr		*P*-Tyr	
	cpm	%	cpm	%	cpm	%
Control	6890	79.7	1295	14.9	455	5.3
Vanadate	6815	68.3	1685	16.3	1665	15.4
Vanadate/control ratio	0.99	0.86	1.30	1.09	3.66	2.89

Membrane protein (1 mg) was incubated for 1 min at 0°C with 1 mM Mn^{2+}/15 μM ATP (25 μCi [γ-^{32}P]ATP) with or without vanadate (30 μM). After precipitation and hydrolysis, phospho amino acids were separated, identified by ninhydrin staining of added phospho amino acid standards, scraped, and quantified. The cpm per mg of protein sample is reported together with the percent that each individual phospho amino acid is of the total phospho amino acid.

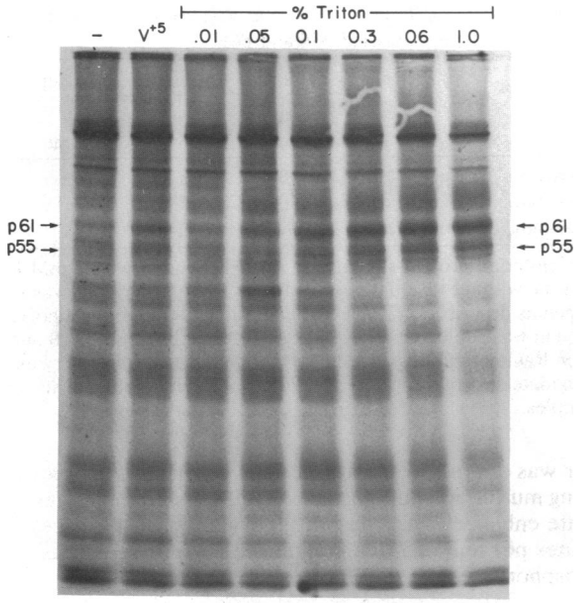

FIG. 2. Effect of nonionic detergent on *in vitro* phosphorylation of Raji cell membranes. Fifty micrograms of protein was phosphorylated as described in Fig. 1, with H_2O control (−), vanadate (30 μM; V^{+5}), or the indicated concentration of Triton X-100. After NaDodSO$_4$/10% PAGE, gels were autoradiographed.

protein bands at M_r 58,000 and 64,000 (p58 and p64) that were resistant to KOH hydrolysis, suggesting phosphorylation of tyrosine residues. Vanadate slightly enhanced the phosphorylation of these two bands.

Splenocytes contained a more complex pattern with four major KOH-resistant phosphoproteins. As in thymocyte membranes, p58 and p64 phosphorylation was only slightly increased by vanadate. In contrast, phosphorylation of p61 and p55 was markedly stimulated by vanadate. A reasonable interpretation is that B cells exhibit vanadate-responsive p61 and p55 phosphorylation and T cells contain two substrates (p64 and p58) whose tyrosine phosphorylation is less dependent on vanadate. In membranes from rat spleen (Fig. 1), p58 and p64 phosphorylation in the presence of vanadate was more difficult to detect than in mouse spleen. Preparation of mouse splenocytes by the three methods used to rid the preparations of erythrocytes—i.e., (*i*) hypotonic lysis, (*ii*) ammonium chloride treatment, or (*iii*) Ficoll-Hypaque gradients—all yielded detectable levels of the putative T-cell bands, p58 and p64. The amount of vanadate-responsive p61 and p55, always substantial, did not vary from preparation to preparation to the extent that p58 and p64 phosphorylation varied.

To understand the heterogeneity in splenocytes, Ig-bearing B cells were purified from spleen cell suspensions using anti-IgG linked to Sepharose. Cells eluted from the affinity matrix were >90% sIg^+. When membranes were prepared and phosphorylated, only p61 and p55 phosphorylation was observed in the presence of either vanadate or Triton (Fig. 4).

To investigate whether or not the difference between T and B lymphocytes was maintained in other populations, several continuous lines derived from transformed mouse T cells were compared to mouse thymocytes and splenocytes. The BALB/c T-cell line, Bd-1, contained bands identical to BALB/c thymocytes (Fig. 5). In both of these T-lymphocyte preparations, phosphorylation of p58 and p64 was only slightly enhanced by vanadate but was stimulated to a greater extent by 0.6% Triton. Two other mouse T-cell lines, EL-4 and RBL-5 (both C57BL/6 strain of origin), also demonstrated p64 and p58 phosphorylation without evidence of p61 and p55 phosphorylation (data not shown).

Comparison of a human T-cell line HSB and the B-cell line Raji, revealed results similar to those of T and B mouse lymphocytes (Fig. 6). Direct analysis of tyrosine phosphorylation was also carried out (Table 2). HSB and Raji cell membranes were phosphorylated in the presence and absence of vanadate. Total membrane phospho amino acid analysis

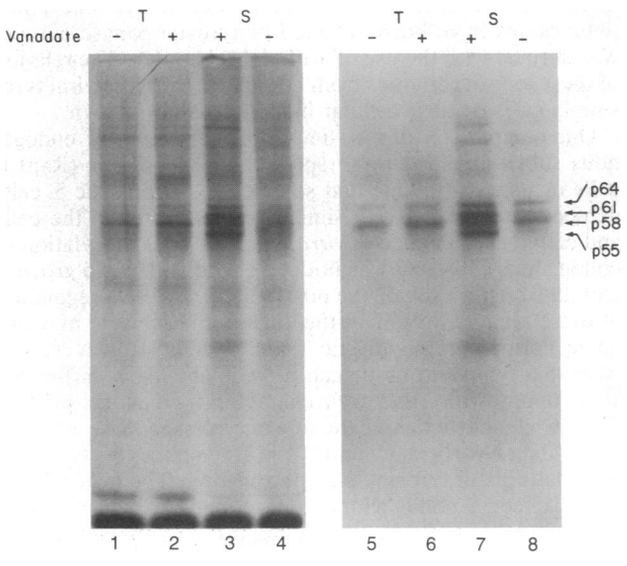

FIG. 3. *In vitro* phosphorylation of thymocyte (T) and splenocyte (S) membranes. Membranes were phosphorylated in the presence of 30 μM vanadate, as described in Fig. 1. Duplicate samples were loaded in lanes 1–4 and 5–8. After NaDodSO$_4$/10% PAGE, the gel was stained, destained, and cut. One-half of the gel (*Right*) was immersed in 2 M KOH for 2 hr, restored to acid destain, and both halves were dried and autoradiographed for 24 hr (*Left*) and 36 hr (*Right*; KOH-treated side).

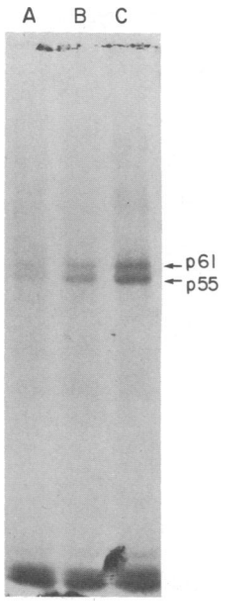

FIG. 4. Phosphorylation of membranes from sIg^+ mouse lymphocytes. Splenocytes were prepared and the Ig^+ cells were purified by affinity chromatography. Membrane fractions were prepared and incubated as described in Fig. 1 with H_2O (lane A), 30 μM vanadate (lane B), or 0.6% Triton X-100 (lane C). The autoradiograph was obtained after NaDodSO$_4$/8% PAGE.

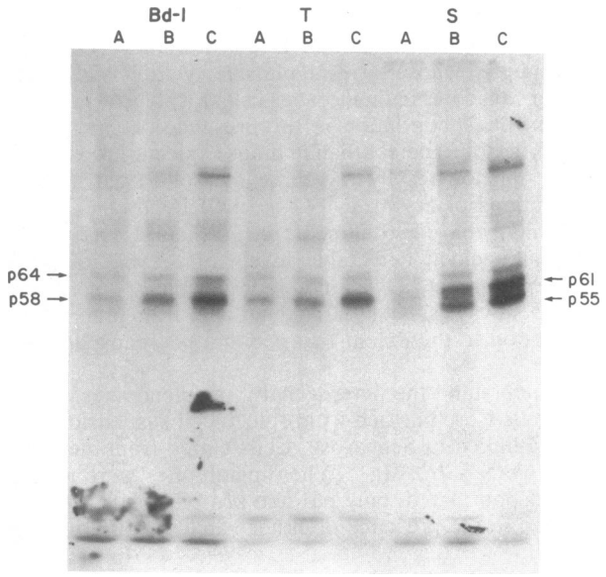

FIG. 5. Phosphorylation of membranes from a BALB/c mouse T-cell lymphoma line (Bd-1), thymocytes (T), or splenocytes (S). Membranes were incubated as described in Fig. 1 with H_2O (lanes A), 30 μM vanadate (lanes B), or 0.6% Triton X-100 (lanes C) followed by $NaDodSO_4$/8% PAGE and autoradiography.

showed that vanadate did not alter *P*-Ser or *P*-Thr significantly in membranes from either cell line. In the T-cell line, vanadate increased total *P*-Tyr by 50%. In Raji membranes, vanadate increased *P*-Tyr 4-fold, as previously reported (18). Specific analysis of the phospho amino acid content of p58 (HSB) and p61 (Raji) was carried out after polyacrylamide gel electrophoresis. Vanadate increased *P*-Tyr content of p61 by >10-fold. In contrast, tyrosine phosphorylation of p58 was only increased by 50%. Again, neither *P*-Ser nor *P*-Thr was altered by vanadate. This confirms our impression using murine splenocytes, T-cell lines, and thymocytes: vanadate enhances tyrosine phosphorylation of the B-cell substrates p61 and p55 to a greater extent than it increases p58 phosphorylation in T cells.

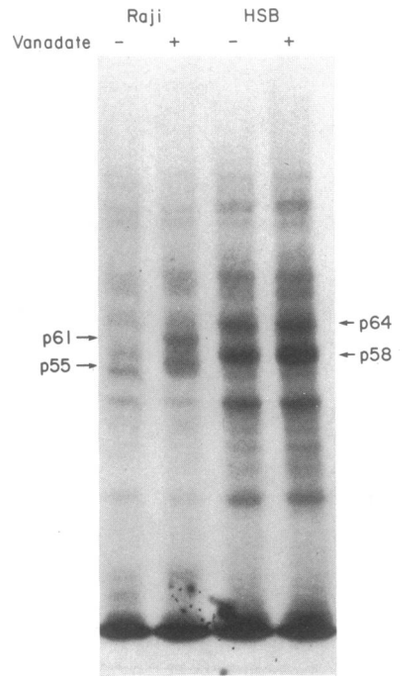

FIG. 6. Phosphorylation of membranes from a human B-lymphoblastoid cell line (Raji) and a T-cell leukemia line (HSB). Membranes were phosphorylated as described in Fig. 1 with (+) or without (−) vanadate (30 μM) followed by $NaDodSO_4$/8% PAGE and autoradiography.

Table 2. *P*-Tyr content of HSB and Raji membranes and electrophoretically purified p58 and p61

	HSB		Raji	
	Total membrane	p58	Total membrane	p61
Control	990	308	375	15
Vanadate	1450	467	1385	210
Vanadate/control ratio	1.46	1.52	3.68	13.9

Membrane protein (1 mg) was phosphorylated with 1 mM Mn^{2+} with or without vanadate (30 μM) and total *P*-Tyr cpm was determined as described in Table 1. In addition, 50 μg of phosphorylated protein was subjected to $NaDodSO_4$/PAGE. p58 from HSB and p61 from Raji membranes were cut out and analyzed for *P*-Tyr content. Vanadate did not markedly alter *P*-Ser or *P*-Thr content in any of the samples.

DISCUSSION

The function and biochemical regulation of tyrosine phosphorylation are largely undefined. The association with growth control is often cited because the majority of proteins exhibiting tyrosine-specific kinase activity have putative growth regulatory properties. Further progress toward understanding the biologic function of these enzymes should be aided by identifying their distribution in normal tissue.

Wong and Goldberg (34) have partially purified a M_r 75,000 tyrosine kinase activity from rat liver cytosol. Dekowski *et al.* (35) reported *in vitro* tyrosine phosphorylation of the erythrocyte membrane band 3 protein. Recently Tuy *et al.* (36), Casnellie *et al.* (37), and Swarup *et al.* (23) have detected tyrosine phosphorylation in erythrocyte membranes and platelet particulate fractions; membranes from normal mouse T cells purified on nylon wool; and rat splenic membranes, respectively. In these studies, tyrosine phosphoproteins have been detected or tyrosine kinase activity was defined with the use of artificial substrates. The relation of these kinase activities from normal tissue to the viral tyrosine kinases or their cellular homologs is not known.

Our comparison of tyrosine phosphorylation of endogenous substrates in normal (splenic) and neoplastic (Raji) B cells or normal (thymic and splenic) and neoplastic T cells demonstrate qualitatively similar results. Thus, in the cells and cell lines examined *in vitro*, tyrosine phosphorylation of endogenous substrate was not correlated with rapid growth. Our data do not exclude the possibility that *in vivo* regulation of these kinases, or in fact the kinases themselves, may differ in normal and neoplastic T and B cells. However, it is clear that lymphoid tissue can provide abundant, differentiated, nongrowing, and well characterized cellular populations in which to examine the role of tyrosine kinase activity.

Our data clearly show that T and B lymphocytes have distinct patterns of tyrosine phosphorylation. The major T-cell band p58 corresponds with that described by Casnellie *et al.* (24). In addition, we describe a larger *P*-Tyr-containing substrate, p64, that has been consistently observed in mouse thymocytes, splenic T cells, and mouse and human neoplastic T-cell lines. It appears that immature, mature, and neoplastic T cells all contain the same two substrates. The B-cell substrates, p61 and p55, are similar in human Raji cells and mouse splenic B cells. They are also observed in rat spleen, which differs from mouse spleen in the lower abundance of p64 and p58 phosphorylation. Swarup *et al.* (23) found two major endogenous rat spleen tyrosine phosphoproteins of M_r

56,000 and 53,000. Since their assays were done in the presence of vanadate, they did not comment on the stimulatory effect of vanadate. In agreement with our findings, they also noted detergent stimulation of tyrosine-specific kinase activity.

While *in vitro* tyrosine phosphorylated substrates are often kinases themselves, it has not been established whether T and B cells contain two distinct kinases. However, we have recently observed that preincubation of mouse splenocyte membranes (21°C, 30 min) with 3 mM *N*-α-tosyl-L-lysyl chloromethyl ketone markedly inhibited the phosphorylation of p64 and p58 (the T-cell bands) while only minimally affecting the phosphorylation of p61 and p55. Since *N*-α-tosyl-L-lysyl chloromethyl ketone is known to inhibit certain tyrosine kinases (38, 39), this result may suggest that T and B cells contain distinct kinases (unpublished results). Whether or not the substrates are kinases remains to be determined, as does whether the substrate pairs (*i*) p64 and p58 and (*ii*) p61 and p55 are separate proteins or whether they are from the same gene product that has been modified post-translationally.

Distinguishing between T- and B-cell populations by nonimmunologic means may be of use in identifying various stages of lymphoid development at which traditional surface markers are not detectable. Moreover, 70% of acute lymphatic leukemias of children are null cell leukemias; i.e., they are not readily typed by standard surface-marker reagents for T and B cells (40). The distinct tyrosine phosphoprotein patterns, the differential response to vanadate, and the potential difference in the T- and B-cell tyrosine kinases may be important in assigning individual null cell leukemias to T- or B-cell lineages. Further work is necessary to establish whether or not these differences in T and B cells will be constant in all neoplastic cells or at earlier or later stages of B-cell development.

The mechanisms by which vanadate and nonionic detergents stimulate tyrosine phosphorylation are unknown; nevertheless, the role of vanadate may be physiologically relevant, because the effective concentrations are achievable in biologic materials (20). Forms of this ion are known to inhibit Na^+/K^+ ATPase and various phosphatase activities, including inhibition of tyrosine dephosphorylation (19–22). Phosphorylation of p61 and p55 was difficult to detect in the absence of vanadate but was stimulated by this compound within 15 sec at 0°C. If vanadate causes *P*-Tyr accumulation by phosphatase inhibition, the ratio of tyrosyl phosphatase to tyrosine-specific kinase in B cells must be extremely high. If this is the case, phosphatase inhibition may be a primary determinant of lymphoid tyrosine phosphorylation. In contrast T-cell substrates are easily detected without vanadate. Stimulation by nonionic detergents occurred without solubilization of the substrate or kinase and eliminated the stimulatory effect of vanadate. The action of detergent also might occur by disrupting coupling between kinase and phosphatase. Other mechanisms by which either vanadate or detergents may directly alter the substrate or kinase resulting in increasing *P*-Tyr may also exist.

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1. Eckhart, W., Hutchinson, M. A. & Hunter, T. (1979) *Cell* **18,** 925–933.
2. Hunter, T. & Sefton, B. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1311–1315.
3. Witte, O. N., Dasgupta, A. & Baltimore, D. (1980) *Nature (London)* **283,** 826–831.
4. Collett, M. S. & Erikson, R. L. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2021–2024.
5. Hunter, T. & Sefton, B. M. (1982) in *Molecular Aspects of Cellular Regulation*, eds. Cohen, P. & Van Heyningen, S. (Elsevier/North-Holland, Amsterdam), Vol. 2, pp. 337–370.
6. Stehelin, D., Varmus, H. E., Bishop, J. M. & Vogt, P. K. (1976) *Nature (London)* **260,** 170–173.
7. Bishop, J. (1983) *Annu. Rev. Biochem.* **52,** 301–354.
8. Carpenter, G., King, L. & Cohen, S. (1978) *Nature (London)* **276,** 409–410.
9. Ushiro, H. & Cohen, S. (1980) *J. Biol. Chem.* **255,** 8363–8365.
10. Hunter, T. & Cooper, J. A. (1981) *Cell* **24,** 741–752.
11. Ek, B., Westermark, B., Wateson, A. & Heldin, C. H. (1982) *Nature (London)* **295,** 419–420.
12. Kasuga, M., Zick, Y., Blithe, D. L., Karlson, F. A., Haring, H. U. & Kahn, C. R. (1982) *J. Biol. Chem.* **257,** 9891–9894.
13. Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983) *J. Biol. Chem.* **258,** 9581–9584.
14. Dasgupta, J. D. & Garbers, D. L. (1983) *J. Biol. Chem.* **258,** 6174–6178.
15. Rubin, R. A. & Earp, H. S. (1983) *Science* **219,** 60–63.
16. Rubin, R. A. & Earp, H. S. (1983) *J. Biol. Chem.* **258,** 5177–5182.
17. Earp, H. S., Rubin, R. A., Austin, K. S. & Dy, R. C. (1983) *Biochem. Biophys. Res. Commun.* **112,** 413–418.
18. Earp, H. S., Rubin, R. A., Austin, K. S. & Dy, R. C. (1983) *FEBS Lett.* **161,** 180–184.
19. Cantley, L. C., Jr., Josephson, L., Warner, R., Tanagasiwa, M., Lechne, C. & Guidotti, G. (1977) *J. Biol. Chem.* **252,** 7421–7423.
20. Ramasarma, T. & Crane, F. L. (1981) *Curr. Top. Cell. Regul.* **20,** 247–301.
21. Swarup, G., Cohen, S. & Garbers, D. L. (1982) *Biochem. Biophys. Res. Commun.* **107,** 1104–1109.
22. Leis, J. F. & Kaplan, N. O. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6507–6511.
23. Swarup, G., Dasgupta, J. D. & Garbers, D. L. (1983) *J. Biol. Chem.* **258,** 10341–10347.
24. Casnellie, J. E., Harrison, M. L., Pike, L. J., Hellstrom, K. E. & Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 282–286.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
26. Peters, C. J. & Theofilopoulos, A. N. (1977) *J. Immunol.* **117,** 1089–1096.
27. Gorer, P. A. (1950) *Br. J. Cancer* **4,** 372–379.
28. Law, L. W. & Appella, E. (1973) *Nature (London)* **243,** 83–87.
29. Foley, G. E., Lazarus, H., Farber, S., Uzman, B. G., Boone, B. A. & McCarthy, R. E. (1965) *Cancer* **18,** 522–529.
30. Gillespie, G. Y., Hansen, C. B., Hoskins, R. G. & Russell, S. W. (1977) *J. Immunol.* **119,** 564–570.
31. Marshak-Rothstein, A., Fink, P., Gridley, T., Raulet, D. H., Bwan, M. J. & Gefter, M. L. (1979) *J. Immunol.* **122,** 2491–2497.
32. Russell, S. W., Gillespie, G. Y., Hansen, C. B. & Cochrane, C. G. (1976) *Int. J. Cancer* **18,** 331–338.
33. Cooper, J. A. & Hunter, T. (1981) *Mol. Cell. Biol.* **1,** 165–178.
34. Wong, T. W. & Goldberg, A. R. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2529–2533.
35. Dekowski, S. A., Rybicki, A. & Drickamer, K. (1983) *J. Biol. Chem.* **258,** 2750–2753.
36. Tuy, F. P. D., Henry, J., Rosenfeld, C. & Kahn, A. (1983) *Nature (London)* **305,** 435–438.
37. Casnellie, J. E., Harrison, M. L., Hellstrom, K. E. & Krebs, E. G. (1983) *J. Biol. Chem.* **258,** 10738–10742.
38. Reichert, N., Davies, P. J. A., Gilbert, J. & Pastan, I. (1979) *Cell* **18,** 369–374.
39. Maness, P. F., Engeser, H., Greenberg, M. E., O'Farrell, M., Gall, W. E. & Edelman, G. M. (1979) *Cold Spring Harbor Symp. Quant. Biol.* **44,** 949–958.
40. Sallan, S. E., Weinstein, H. J. & Nathan, D. G. (1981) *J. Pediatr.* **99,** 676–688.