Vanadate stimulates tyrosine phosphorylation of two proteins in Raji human lymphoblastoid cell membranes

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A membrane fraction from Raji human lymphoblastoid cells exhibited tyrosine-specific kinase activity. Vanadate increased tyrosine phosphorylation up to 5-fold; serine and threonine phosphorylation were unchanged. The stimulation was detectable within 15 s at 0°C and at concentrations of vanadate (0.3 and 1.0 μM) present in normal tissues and blood. The tyrosine phosphorylation of two substrates, M_1 61000 and 55000, was dependent upon vanadate and incorporation into these substrates represented the majority of the vanadate-sensitive tyrosine phosphorylation.

Vanadium *Lymphocyte* *Protein phosphorylation* *Membrane protein* *Tyrosine-specific kinase*

1. INTRODUCTION

A new class of tyrosine-specific protein kinases has been identified [1]. One subgroup of these enzymes is typified by the transforming gene products of several retroviruses [1–4] (e.g., the src kinase of Rous sarcoma virus). A great deal is known about the genes that code for these proteins, but little is known about their biochemical regulation. In contrast, tyrosine phosphorylation of the cell surface receptors for growth factors (EGF [5], PDGF [6] insulin, [7] and somatomedin C [8]) is clearly regulated by binding of an appropriate ligand to the receptor.

The biological and biochemical effects of the trace element vanadium have received increased attention since the discovery that vanadate inhibits Na^+/K^+ ATPase activity [9–11]. In cells, vanadate mimics the action of insulin [12] and enhances the effects of EGF [13]. In biochemical studies, vanadate inhibits ATPase and phosphatase activities [9–11,14]. We here report that membranes from a human lymphoblastoid cell line derived from Burkitt's lymphoma [15] contain a tyrosine-specific kinase activity; vanadate markedly stimulates the tyrosine phosphorylation of two Raji membrane proteins.

Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; p-Tyr, p-Ser, p-Thr, phosphotyrosine, phosphoserine and phosphothreonine

2. MATERIALS AND METHODS

Raji cells were grown to a density of 10^6 cells/ml. Cells were centrifuged, washed with 0.15 M NaCl and resuspended in 10 mM phosphate buffer, pH 7.0/1 mM EDTA. After disruption with Brinkman Polytron apparatus, homogenates were centrifuged at $1000 \times g$. The supernatant was centrifuged at $105000 \times g$, the pellet was resuspended in 20 mM Pipes buffer (pH 7.0) and the protein concentration was determined [16].

Membranes were phosphorylated in 30 mM Mg^{2+} or 1 mM Mn^{2+}, 50 mM Pipes buffer (pH 7.0) at 0°C with 1–15 μM [γ-^{32}P]ATP in the presence or absence of vanadate (Na_3VO_4, Fisher Scientific). For analysis of phosphoamino acid

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content, the reaction was stopped after 1 min at 0°C with 50% trichloroacetic acid. The precipitates were collected, washed with 10% trichloroacetic acid followed by ether/ethanol, dried, hydrolyzed in 6 N HCl (3 h at 110°C) and separated by a two-dimensional procedure [3]. Specific phosphorylation was examined by autoradiography on Kodak XAR-5 film after electrophoresis of phosphorylated proteins on 8 or 10% polyacrylamide gels as in [17].

3. RESULTS AND DISCUSSION

Phosphorylation at 0°C of crude membranes was followed by phosphoamino acid separation and analysis of eluted phosphoamino acid spots by liquid scintillation counting; p-Tyr constituted 2% of the basal phosphoamino acid. Vanadate (30 μM) increased total p-Tyr content of membrane proteins 5-fold (fig.1A); p-Ser and p-Thr were not altered. The lowest concentrations (0.3 and 1.0 μM) resulted in a doubling and tripling of p-Tyr. Vanadate increased p-Tyr by 15 s, the earliest time point tested (fig.1B). The effect of vanadate, which was observed at ATP concentrations ranging from 1–15 μM was not explained by alterations in ATP availability. After a 0°C, 1 min incubation, vanadate (0.3–30 μM) did not alter the proportion of [^{32}P]ATP recovered. In both control and vanadate-stimulated reactions, about 75% (range 71–77%) of the radioactivity recovered after perchloric acid precipitation, neutralization, and thin-layer chromatography [17] was [^{32}P] ATP.

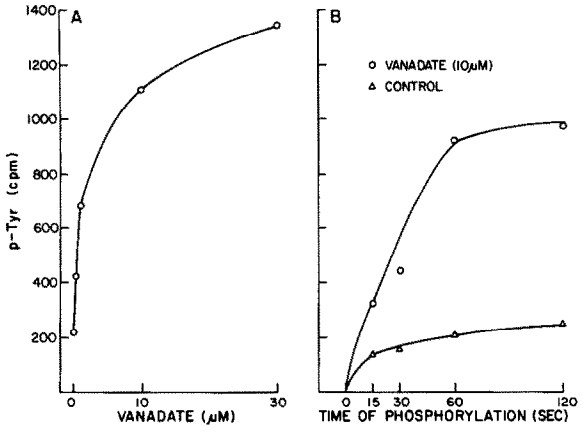

Fig.1. Concentration- and time-dependence of vanadate-stimulated p-Tyr accumulation. One mg of membrane fraction was preincubated for 10 min at 0°C in 50 mM Pipes/10 mM phosphate buffer (pH 7.0), 30 mM Mg_2Cl, with 0–30 μM vanadate; 15 μM ATP (25 μCi [γ-^{32}P]-ATP/tube) was added. After 1 min at 0°C, the reaction was terminated and the phosphoamino acids separated. Autoradiography was performed by exposing the chromatogram to Kodak XAR-5 film at −70°C for two days. The phosphoamino acid spots, identified by ninhydrin staining of added standards, were scraped, eluted and counted. (A) Concentration-dependence; (B) Time-dependence–10 μM vanadate, 0°C.

SDS–polyacrylamide gel electrophoresis and autoradiography showed that two proteins (estimated M_r values 61000 and 55000, p61 and p55) were preferentially phosphorylated in the presence of 30 μM vanadate (fig. 2b,d,f). Phosphorylation was stimulated by vanadate in the presence of either 30 mM Mg^{2+} (lanes a,b) or 1 mM Mn^{2+} (lanes e,f). Two techniques demonstrated that the increase in p61 and p55 phosphorylation occurs predominantly on tyrosine residues. The phosphate remained after gels were treated with 2 M KOH, a technique which takes advantage of the relative alkali stability of p-Tyr [18] (fig. 2c,d). In a separate experiment, membranes were phosphorylated in 30 mM Mg^{2+} and electrophoresed. p61 and p55 were identified by the Coomassie blue staining pattern, cut from the wet gel, eluted from gel slices electrophoretically, precipitated and hydrolyzed, and their phosphoamino acid content was determined [17]. Vanadate (10 μM) increased the p-Tyr content of p61 4-fold and of p55 2.3-fold. Slight enhancement of phosphorylation of a third protein (M_r 25000) was noted but the phosphate incorporated was alkali-labile.

Stimulation of p61 phosphorylation was detected at concentrations as low as 0.3 and 1.0 μM while increased p55 phosphorylation was seen at higher concentrations (fig.3). The concentration-dependence roughly paralleled the incorporation into total membrane p-Tyr. The time course of p61 and p55 phosphorylation also parallels that of total membrane p-Tyr with increases apparent by 15 s at 0°C (fig.4). Electrophoresis on an 8% polyacrylamide gel demonstrated another point. The basal phosphorylation in the region of p61 seen on 10% gels is in another protein distinct from p61; in other words, there is very little

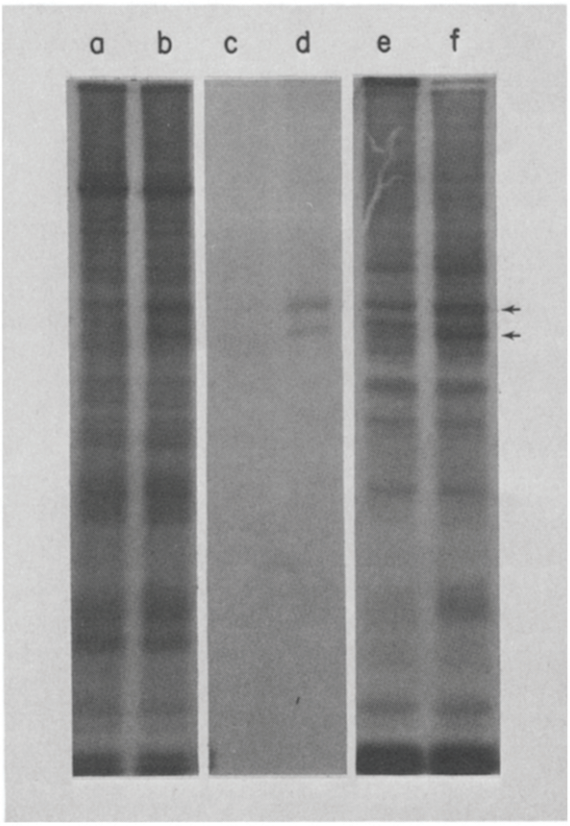

Fig.2. Effect of vanadate on phosphorylation of individual membrane proteins. 50 μg membrane protein was preincubated for 10 min at 0°C with 30 mM $MgCl_2$ (a–d) or 1 mM $MnCl_2$ (e,f), plus vanadate (30 μM, b,d,f). After a 1 min, 0°C incubation with 1 μM ATP (5 μCi [γ-^{32}P]ATP), the reaction was stopped and electrophoresis was performed. Lanes a–d were run on the same 10% polyacrylamide gel, which was stained and destained. Lanes c,d were then treated with 2 M KOH for 2 h at 55°C. The swollen gel was then rewashed with destain solution until it was about the original size. Vanadate increased the phosphorylation of p61 (upper ←) and p55 (lower ←); the phosphate was alkali-resistant.

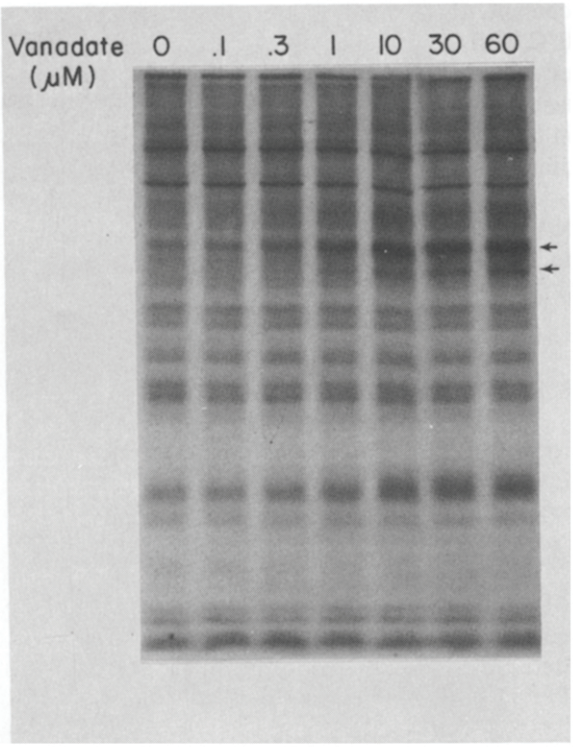

Fig.3. Concentration-dependence of vanadate stimulation of p61 (upper ←) and p55 (lower ←) and p55 phosphorylation; 50 μg membrane protein was phosphorylated with 30 mM Mg^{2+}, 1 μM ATP and 0–60 μM vanadate, and run on a 10% polyacrylamide gel.

phosphate incorporated into either p61 or p55 in the absence of vanadate.

Raji lymphoblastoid cells contain tyrosine-specific kinase activity. Other tyrosine kinases that are not associated with growth factor receptors may also be located on membranes; their endogenous substrates have not been well defined. It is unresolved whether these enzymes autophosphorylate or serve as a substrate for other tyrosine kinases [1]. Thus, vanadate may promote autophosphorylation of two membrane kinases, p61 and p55. Alternatively, the catalytic activity may reside in either p61 or p55 or in a separate protein. It is also possible that p55 is a proteolytic product of p61. A mouse lymphoma line, LSTRA, contains a distinct tyrosine phosphoprotein of M_r 58000 [19].

The present studies do not definitively elucidate the mechanism by which p-Tyr accumulation is promoted. Three possibilities exist:

(i) Vanadate inhibits p-Tyr dephosphorylation. It has been observed [14] that vanadate inhibited a p-Tyr phosphatase activity of human A431 cells. Fig. 2 and 4 demonstrate that, under these conditions, tyrosine phosphorylation of p61 and p55 is difficult to detect in the absence of vanadate but is increased within 15 s at 0°C in the presence of vanadate. If the effect of

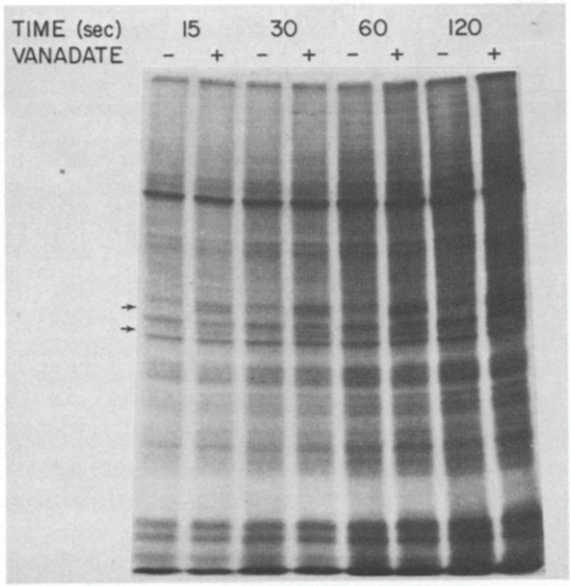

Fig.4. Time-dependence of vanadate stimulation of p61 (upper ←) and p55 (lower ←) and p55 phosphorylation. 50 μg protein was phosphorylated with 1 mM Mn^{2+}, 1 μM ATP in the presence or absence of 30 μM vanadate. The reaction was stopped at the indicated times after the addition of labeled ATP.

vanadate is due to inhibition of tyrosine dephosphorylation, the phosphatase must be present in vast excess and be very active at 0°C;

(ii) Vanadate directly stimulates kinase activity. As discussed above, the molecular mass of the protein kinase remains to be determined;

(iii) Vanadate forms complexes with protein [20], and could in some manner change substrate conformation to one more favorable for tyrosine phosphorylation. Modification of a tyrosine residue within the active site of phosphoglucomutase by vanadate-enzyme interaction has been postulated as one mechanism by which vanadate markedly stimulates this phosphotransferase activity [21].

While further experiments will be necessary to understand its mechanism of action, vanadate at physiologic concentrations is a potential regulator of membrane tyrosine phosphorylation. The tyrosine phosphorylation of membrane substrates in mouse [19] and human lymphoproliferative disorders may be pathogenetically significant or may simply reflect the tissue of origin.

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