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Numerous proteins phosphorylated on tyrosine and enhanced tyrosine kinase activities in vanadate-treated NIH 3T3 fibroblasts

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A monoclonal antibody that can immunoprecipitate proteins containing phosphotyrosine has been isolated and characterized. To identify proteins that can act as substrates for tyrosine kinases in intact cells, extracts of phosphate-labeled NIH cells that had been treated with the phosphotyrosyl phosphatase inhibitor, vanadate, were precipitated with the antibody, and the immunoprecipitates were analyzed by two-dimensional gel electrophoresis. Numerous proteins were specifically precipitated from vanadate-treated NIH 3T3 cells by the antibody. The high level of phosphotyrosine detected in vanadate-treated cells is presumably primarily due to phosphatase inhibition, but approx. 2-fold increased tyrosine kinase activities were also detected in extracts of the cells after treatment with vanadate. The enhanced tyrosine kinase activity may contribute to the generation of the transformed phenotype seen in response to treatment with vanadate.

Introduction

Much evidence has accumulated to suggest that tyrosine phosphorylation is involved in growth regulation of eukaryotic cells. Many growth factor receptors are associated with tyrosine kinases that are activated upon binding their ligands. Also, about half of the known oncogene products are associated with tyrosine kinase activities (for reviews see Ref. 1). This has led to speculations that oncogenes and growth factors may act on similar signaling pathways in cells. Direct support for this view has recently been provided by the findings that some oncogenes code for proteins that are related to components of the signaling pathways for known growth factors [2–5]. The receptors for these factors are associated with tyrosine kinase activities [6–8].

It is therefore of interest to identify proteins that can be phosphorylated on tyrosine in intact cells. To facilitate this, we have produced a monoclonal antibody, P-tyr-1, that reacts with phosphotyrosine. In this study we have examined the proteins that are phosphorylated on tyrosine in intact cells in response to treatment with vanadate, an inhibitor of phosphatases acting on phosphotyrosine [9–12]. Addition of vanadate to the tissue culture medium of normal fibroblasts results in up to 40-fold elevation of the cellular content of phosphotyrosine and to cell transformation [13].

Presumably, effects of vanadate are primarily mediated by inhibition of dephosphorylation of phosphotyrosine. However, since some tyrosine kinases are activated by phosphorylation on certain tyrosine residues [14–18], we examined

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whether the overall tyrosine kinase activity in the cells might be elevated by vanadate treatment.

Materials and Methods

Isolation of the P-tyr-1 hybridoma

Aminobenzyl phosphonate, a structural analogue of the side-chain in phosphotyrosine, coupled to hemocyanin, has previously been shown to be useful as an immunogen to generate antibodies reactive to phosphotyrosine in proteins [19]. The conjugate (ABP-HC) was prepared by diazotization of aminobenzylphosphonate followed by coupling to hemocyanin [19]. The conjugate contained 1 molecule of aminobenzyl-phosphonate per approx. 6 kDa carrier protein as determined by phosphate analysis. 3–4 month old female BALB/c mice were immunized subcutaneously with 0.1 mg ABP-HC in Freund's complete adjuvant, boosted intraperitoneally 4 weeks later with 0.1 mg ABP-HC in Freund's incomplete adjuvant. The spleens were used 3 days after the boost for fusion with X63-Ag 8.6.5.3 myeloma cells with poly(ethyleneglycol), relative molecular mass 1500 kDa (BDH), according to previously described procedures [20]. Hybrids were selected by growth for 14 days in HAT medium in microtiter wells (NUNC). Screening for hybridomas producing antibodies reactive with ABP-HC was performed by coating microwell plates (NUNC) with the antigen by incubation with 1 μg/ml ABP-HC overnight, incubating with the supernatants, and assaying for bound antibody by binding of ^{125}I-labeled protein A. Seven wells in one experiment were found to contain hybridomas that produced antibodies reactive to ABP-HC by this procedure. The specificity of the binding was preliminarily assessed by incubation in the presence of 10 mM nitrophenyl phosphate, a compound that has obvious structural similarity to the side-chain in phosphotyrosine. The binding of antibodies from four of the wells was competed by nitrophenyl phosphate. Antibodies produced by cells from one of these wells precipitated phosphate-labeled proteins from XC cells (a rat tumor induced by Rous sarcoma virus [21]) only in the absence of nitrophenyl phosphate. The seven most prominent precipitated proteins were all found to contain phosphotyrosine by two-dimensional phosphoamino acid analysis [22]. The hybridomas were cloned twice and the antibody, P-tyr-1, was characterized further as described in the Results section.

Purification of the P-tyr-1 antibody

For all the experiments reported here, the P-tyr-1 antibody was purified by affinity chromatography: 70 mg aminobenzyl phosphonate was coupled to 20 ml cyanogen bromide-activated Sepharose (Pharmacia). Ascites was diluted with an equal volume of 20 mM $NaHCO_3$ (pH 8.0), 1 mM EDTA, 100 mM NaCl, and applied to the affinity column after clarification by centrifugation. After washing, bound antibody was eluted in the same buffer containing 5 mM nitrophenyl phosphate, and the preparation was dialyzed extensively before use. For immunoprecipitation, the purified antibody was coupled to cyanogen bromide-activated Sepharose; approx. 6 mg protein was bound per ml beads.

Competition assays

ABP-HC was bound overnight at 0.25 μg/ml to microwell plates in 20 mM borate pH 8.0 (all incubations were 55 μl per well). Unsaturated binding-sites were blocked by incubation with N2TE (100 mM NaCl, 20 mM Tris (pH 8.0), 1 mM EDTA) containing 1% Tween 20, and, after washing, 0.5 μg/ml purified P-tyr-1 antibody was added in N2TE with the relevant concentrations of competitors and incubated overnight on a rotatory shaker. The plates were washed and subsequently incubated with 100 ng ^{125}I-labeled protein A per well. This dose of protein A very nearly saturated P-tyr-1 bound in the absence of competitor. The following day the plates were washed, and the individual wells were counted in a gamma counter.

Tissue culture, cell labeling and immunoprecipitation

The NIH 3T3 cells were the clone NIH-6 Cl 32 that has previously been described [13]. The cells were grown in Dulbecco's modified Eagle's medium containing 5% newborn calf serum. The cells were regularly tested for mycoplasma contamination.

The cells were seeded in 5 cm petri dishes. Labeling was performed when the cultures were

subconfluent by replacing the culture medium with 200 μl medium containing 1/10 of the normal concentration of phosphate and 1–12 mCi $^{32}P_i$. The higher activities were used in experiments intended for phosphoamino acid analysis and in experiments that did not include vanadate during the labeling period. Immunoprecipitation was performed as described [13]. In some experiments, 5 mM ATP was added to the lysis buffer. This did not influence the results noticeably, indicating that the phosphorylations were not artefacts generated by kinase reactions occurring in the cell extracts [22].

Two-dimensional gel electrophoresis was performed as described by O'Farrell [23] with the modifications of Bravo et al. [24] using 13% acrylamide in the second-dimension gel. The immunoprecipitates were dissolved directly in the first-dimensional gel buffer before loading. To facilitate comparisons of different gels unlabeled extracts of NIH cells were included in the sample buffer, and the gels were stained with Coomassie blue. The pH in the first dimension was estimated by slicing gels run in parallel, eluting the ampholytes in approx. 6 vol 20 mM KCl, and measuring the pH.

Phosphoamino acid analysis

The presence of phosphotyrosine in the proteins in the two-dimensional gels was demonstrated by the resistance of the signals to alkaline hydrolysis [25], and, more rigorously, by one-dimensional thin-layer electrophoresis of eluted protein, as described [13]. In addition, material from eight prominent spots was analyzed by two-dimensional electrophoresis at pH 1.9 and 3.5 of hydrolyzates [22] and phosphotyrosine was found in all of these. Material from the total cell extracts was analyzed by two-dimensional electrophoresis after phenol extraction of total cell protein [22].

Assay of tyrosine kinase activities

Extracts were prepared from subconfluent cell cultures after three rapid washes with 20 mM Hepes (pH 7.2), 100 mM NaCl at 0 °C by addition of the same buffer containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 0.2 mM sodium orthovanadate. The extracts were clarified twice by centrifugation for 5 min at 20 000 × *g*, dialyzed 4–6 h to remove endogenous ATP that might interfere with the subsequent kinase assays, clarified once more, and the protein concentration was adjusted to 5 mg/ml.

The phosphorylation assays were initiated by mixing extracts with an equal volume of 10 μM ATP, 10–20 μCi gamma [^{32}P]ATP, 10 mM $MnCl_2$, 4 mg/ml [val]^5-angiotensin and incubating at 30 °C. Aliquots were taken at different times and transferred to 2% trichloroacetic acid (final concentration). After removal of precipitated material by centrifugation, the radioactivity incorporated into angiotensin was determined by the filter assay of Wong and Goldberg [26]. The incorporation was linear for at least the first 20 min, and the rates of phosphorylation were calculated from this period. We also note that the incorporations were linear with respect to protein concentration up to the amount of protein used in the assays.

Results

Characterization of the P-tyr-1 antibody

A hybridoma, P-tyr-1, producing antibodies reactive to aminobenzyl phosphonate conjugated to hemocyanin (ABP-HC) [19] was isolated as described in detail in the Materials and Methods section. To characterize the specificity of the antibody, binding of P-tyr-1 to ABP-HC was measured by a radioimmunoassay in the presence of various competitors. As is evident from Table I, ABP-HC was a powerful competitor, whereas unconjugated hemocyanin was not. Furthermore, three compounds that contained the phenyl phosphate group (nitrophenyl phosphate, phenyl phosphate and phosphotyrosine) as well as aminobenzylphosphonate competed significantly for antibody binding. This strongly indicates that the phenyl phosphate moiety in phosphotyrosine is part of the epitope recognized by the antibody.

In contrast, substances having little structural similarity to phosphotyrosine did not compete for antibody binding. Importantly, phosphoserine and phosphothreonine did not compete. The lack of competition by nitrophenyl sulfate was remarkable, since the analogue, nitrophenyl phosphate, was an efficient competitor. This agrees with the observation that we have consistently been unable to precipitate sulfate-labeled proteins from the XC

TABLE I

COMPETITION OF BINDING TO THE P-Tyr-1 ANTIBODY

ABP-HC was bound to microwell plates, P-tyr-1 antibody was added with various concentrations of competitors and the amount of bound of P-tyr-1 was quantitated by binding of ^{125}I-labeled protein A, as described in Materials and Methods.

Competitor	Concentration at 50% competition
ABP–HC	0.00082 mg/ml
Hemocyanin	> 2 mg/ml
Phosphotyrosine	0.41 mM
Phosphothreonine	> 20 mM
Phosphoserine	> 20 mM
nitrophenyl phosphate	0.041 mM
Nitrophenyl sulfate	> 20 mM
Nitrophenol	> 20 mM
Phenyl phosphate	2.0 mM
Aminobenzyl phosphate	0.028 mM
Phosphate	20 mM
Sulfate	> 20 mM

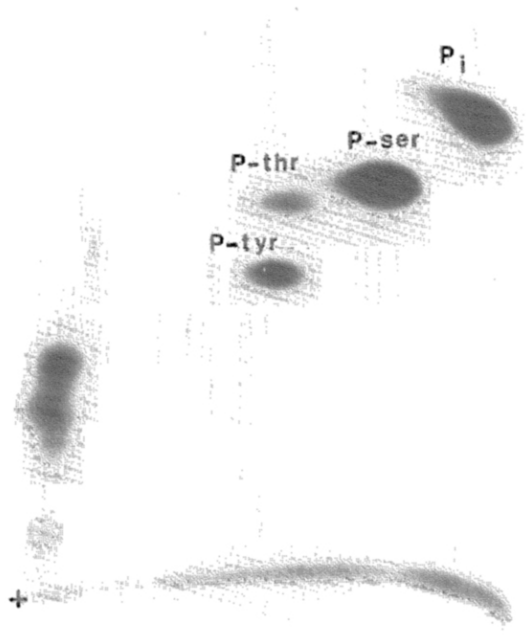


Fig. 1. Phosphoamino acid analysis of protein extracted from vanadate-treated cells. NIH cells were labeled with $^{32}P_i$ for 4 h in the presence of 0.5 mM vanadate. The total cell protein was extracted with phenol [22], and partially hydrolyzed with 6 M HCl at 110 °C for 2 h. The hydrolysate was applied at the site indicated by + on a cellulose thin-layer plate, and electrophoresis was performed at pH 1.9 in the horizontal direction and at pH 3.5 in the vertical direction. The plates were subsequently subjected to autoradiography. Non-radioactive phosphoamino acids were included as markers.

cells [27], indicating that P-tyr-1 does not have significant affinity for tyrosine sulfate in proteins.

It was of interest to quantitate the overall efficiency of precipitation of phosphotyrosine from cell extracts. As described in Table II, about half of the phosphotyrosine was removed from the XC cell extracts by one round of incubation with the P-tyr-1 antibody, but little extra was removed by a second round of incubation. Incomplete precipitation of the phosphotyrosine present in cell lysates seems to be a general problem with anti-phosphotyrosine antibodies [28].

To complete the description of the P-tyr-1 antibody, we finally note that it has been found useful in Western-blotting procedures.

TABLE II

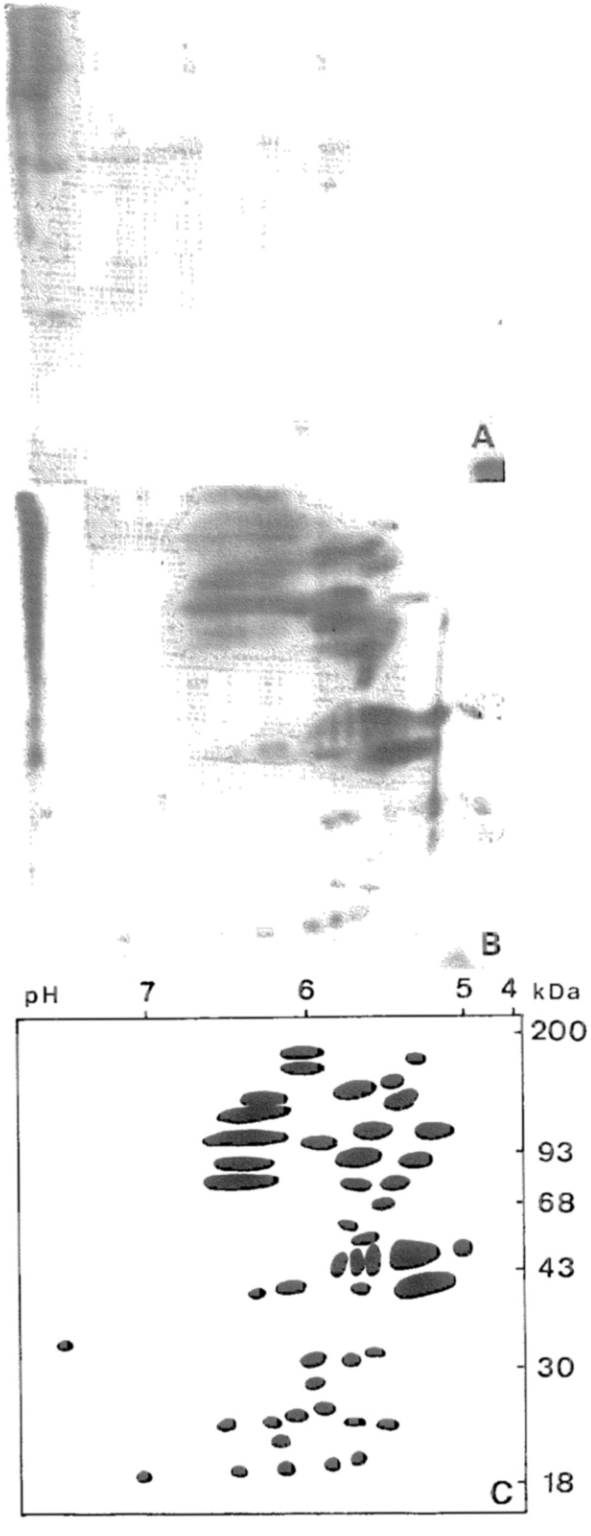
REMOVAL OF PHOSPHOTYROSINE FROM XC CELL EXTRACTS BY INCUBATION WITH P-tyr-1

XC cells were labeled with ^{32}P for 4 h, and the standard RIPA buffer extract [13] was prepared. Total protein was isolated by phenol extraction [22] from aliquots taken before, and after one or two rounds of incubation with the P-tyr-1 antibody. ^{32}P in total protein was not detectably reduced by the incubations. The amounts of label in the three phosphoamino acids were determined after separation of the hydrolyzed protein by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 and are given as percentages.

	Total extract	Extract after one precipitation	Extract after two precipitations	First precipitate
Phosphotyrosine	2.7	1.4	1.2	39
Phosphoserine	84	87	88	56
Phosphothreonine	13	12	11	6

Proteins phosphorylated on tyrosine in vanadate-treated cells

In the subsequent studies we were interested in examining a cell line in its untransformed state and after treatment with vanadate, and NIH 3T3 cells were chosen as the object of these studies. In analyzing the proteins that are phosphorylated on tyrosine in response to vanadate, it was clearly



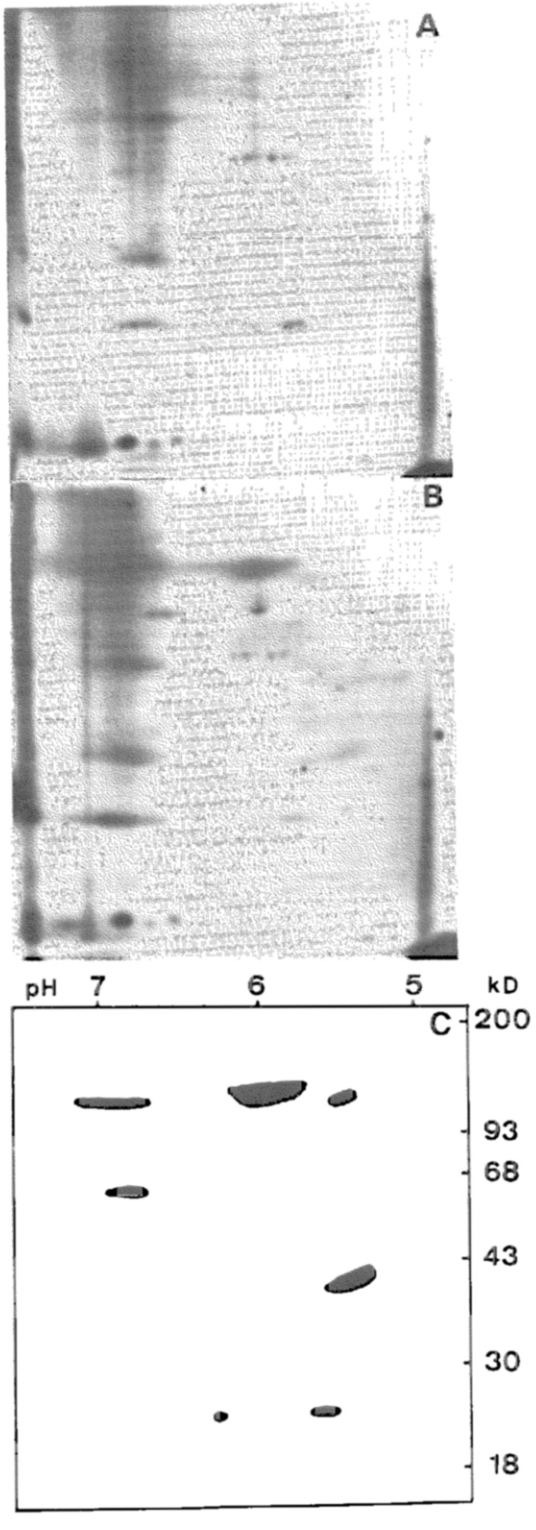
advantageous to obtain the highest possible amount of label in phosphotyrosine. Since the turnover of the phosphate group in phosphotyrosine is rapid [29] we chose 4 h labeling periods. Furthermore, vanadate was added at a high concentration (0.5 mM) during the labeling periods. This dose was toxic to the cells if they were kept with the vanadate for prolonged periods of time. However, the cells were impermeable to trypan blue after the 4 h labeling periods, and the cultures survived provided that they were shifted to vanadate-free medium.

As a consequence of this procedure very high levels of ^{32}P were incorporated into phosphotyrosine. In three separate experiments the levels of radioactivity incorporated into phosphotyrosine were 15, 12 and 11% of the total radioactivity incorporated into the three analyzed phosphoamino acids (phosphotyrosine, phosphoserine and phosphothreonine) as determined by partial acid hydrolysis and subsequent two-dimensional thin-layer electrophoresis (Fig. 1).

Numerous proteins were specifically precipitated from extracts of vanadate-treated cells by the P-tyr-1 antibody (Fig. 2). There was some variation in the relative intensities of the signals from the proteins in different experiments, as might be expected in this type of analysis. However, the overall pattern was consistent, and in Fig. 2C the results from seven independent experiments are summarized. Only proteins that were reproducibly observed and in which, furthermore, phosphotyrosine was directly demonstrated by partial acid hydrolysis and subsequent thin-layer electrophoresis are indicated (data not shown). Most of the larger proteins were additionally found to contain aliphatic phosphoamino acids.

To determine whether the high level of ^{32}P incorporated into these proteins reflect increased levels of phosphorylation on tyrosine, the NIH

Fig. 2. Proteins precipitated by the P-tyr-1 antibody from NIH cells labeled with 2 mCi $^{32}P_i$ in the presence of 0.5 mM vanadate for 4 h. (A) Precipitation in the presence of 15 mM nitrophenyl phosphate. (B) Precipitation without the competitor. In B, the gel was exposed to autoradiography for one day with an intensifying screen, and in A was exposure was twice as long. (C) Localization of proteins that were shown to contain phosphotyrosine by phosphoamino acid analysis.



cells were labeled for 26 h in order to approach isotope equilibrium, and 0.5 mM vanadate was added for the last 4 h of incubation. The resulting pattern of immunoprecipitated proteins was essentially identical to that seen in Fig. 2, indicating that vanadate does indeed induce an increase in the absolute level of phosphorylation of these proteins (data not shown).

Also, a pattern of proteins similar to that illustrated in Fig. 2 was detected in immunoprecipitates of NIH cells transformed by growth for 4 days in 18 μM vanadate. Thus, these phosphorylations also appear to occur at non-toxic doses of vanadate.

In contrast, only weak signals were obtained by precipitation of extracts of NIH cells that were not treated with vanadate (Fig. 3). In the most prominent protein (120 kDa, focusing at pH 6.0) only phosphoserine and phosphothreonine was detected. This is possibly ATP citrate lyase; anti-phosphotyrosine antibodies appear to react with ATP citrate lyase by binding to the phosphohistidine that is present in this protein (Ref. 28 and data not shown). Phosphotyrosine was detected in the 115 kDa protein focusing around pH 7.0. Five other proteins were in addition reproducibly precipitated from untreated cells (Fig. 3), but too little radioactivity was incorporated to allow definite analysis of the phosphoamino acid content. By carefully comparing the positions of these proteins with Coomassie stained non-radioactive marker proteins included in the gels (see Materials and Methods), the localizations of four of these proteins, the three lower molecular weight species and the 120 kDa protein focusing at pH 5.4, were found to correspond to positions of proteins that were found to contain phosphotyrosine in vanadate-treated cells.

Fig. 3. Protein precipitated from NIH cells in the absence of vanadate. Cells were labeled for 4 h with 12 mCi $^{32}P_i$. (A) Precipitation in the presence of 15 mM nitrophenyl phosphate. (B) Precipitation without the competitor. (C) Proteins that were specifically precipitated by the P-tyr-1 antibody as compiled from five separate experiments. The gels were exposed for 14 days with intensifying screens.

Tyrosine kinase activities in cells treated with vanadate

Tyrosine kinase activities were measured in Triton X-100 cell extracts using [val]^5-angiotensin as an exogenous substrate [26]. In four independent experiments, extracts of NIH cells that had been treated for 4 h with 0.5 mM vanadate were found to phosphorylate angiotensin at 1.7–3.0-fold faster rates than comparable controls. Also, NIH cells that were transformed by growth for 3 days in 18 μM vanadate exhibited 1.5- and 1.7 fold-increases in two separate experiments. The enhanced rates of phosphorylation of angiotensin did not appear to be due to differences in phosphatase activities in the extracts, because 0.2 mM vanadate was added to the extraction buffer and control experiments showed that dephosphorylation of angiotensin was effectively blocked (data not shown).

An important question was whether the enhanced tyrosine kinase activity might be due to increased synthesis of kinase molecules in response to vanadate. The tyrosine kinase activities were therefore measured after suppression of protein synthesis by cycloheximide before vanadate was added to the cell cultures (Table III). Three independent experiments were performed with similar results. The high concentrations of vanadate used in these experiments inhibited protein synthesis as has previously been reported [30], but protein synthesis was more effectively blocked by addition of cycloheximide. The reason for the slight increase in angiotensin phosphorylating activity that was measured in the cells treated with cycloheximide is not clear. However, tyrosine kinase activity was enhanced to a similar degree by vanadate when protein synthesis was inhibited by the drug. This observation argues against the possibility that the increase in tyrosine kinase activity could be due to increased production of tyrosine kinase molecules in response to vanadate.

TABLE III

TYROSINE KINASE ACTIVITIES IN EXTRACTS OF NIH CELLS TREATED WITH VANADATE

Half of the cultures were incubated for 32 h without serum before the start of the experiment and, where indicated, 0.5 mM cycloheximide was added 15 min before addition of 0.5 mM vanadate. The cultures were then incubated for 4 h, lysed in Triton X-100, and the tyrosine kinase activities were assayed. To determine the amount of protein synthesis in the incubation period, [^{35}S]methionine (0.2 mCi/ml) was added to triplicate parallel cultures at the time of addition of vanadate. The cells were then washed, lysed in SDS, and the trichloroacetic acid precipitable counts were determined. After correction for protein content in the samples, the relative incorporations were calculated, setting the incorporation in untreated cultures to 100%.

Presence of			Tyrosine kinase activity (pmol phosphate incorporation per mg protein per min)	Protein synthesis (%)
serum	cyclo heximide	vanadate		
+	−	−	0.50	100
+	−	+	0.79	39
+	+	−	0.55	1.4
+	+	+	0.99	1.3
−	−	−	0.31	18.7
−	−	+	0.55	11.7
−	+	−	0.33	1.1
−	+	+	0.60	1.8

One possible mechanism for activation of the tyrosine kinases is that receptors on the cell surface might acquire increased affinity for factors present in the serum and thereby activate associated tyrosine kinases. This, however, seems unlikely given the observation that the tyrosine kinase activity was also enhanced by vanadate in the absence of serum (Table III).

Discussion

Much work has been devoted to identifying the cellular substrates for the tyrosine kinases (for review see Ref. 31) on the reasonable assumption that phosphorylation of certain cellular proteins on tyrosine is involved in growth regulation. One difficulty is that only relatively low specific activities can be obtained by labeling cells with phosphate because of the high intracellular concentration of ATP. With the often used technique involving alkaline hydrolysis to enhance the signal from ^{32}P-labeled phosphotyrosine relative to that from phosphoserine, the level of detection has been estimated to correspond to 50000–100000 phosphorylated protein molecules per cell [32]. Unfortunately, there is no reason to believe that the functionally important phosphorylations should be quantitatively prominent ones.

By including 0.5 mM vanadate in the labeling medium, a high level of radioactivity was incorpo-

rated into phosphotyrosine. Phosphotyrosine was detected in 47 protein spots in two-dimensional gels of immunoprecipitates. Obviously, some of these spots may represent different modifications of identical polypeptides which would lead to an overestimation of the number of polypeptides that can be phosphorylated on tyrosine. However, numerous proteins that gave rise to signals that were too weak to allow phosphoamino acid analysis were reproducibly precipitated by the antibody. Furthermore, proteins that contain phosphotyrosine may not have been detected if they are not precipitated by the antibody or if they are insoluble in the extraction buffer. Thus, we believe that the actual number of cellular proteins that can be phosphorylated on tyrosine may be in the order of several hundreds.

An important question is whether the phosphotyrosine-containing proteins that are found in vanadate-treated cells represents an enhancement of signals from proteins that can be phosphorylated on tyrosine under physiological conditions, but only at levels that are too low to allow their detection, or whether the appearance of these proteins represent an experimental artifact induced by vanadate. We note that normal cells do seem to contain a large number of proteins that are phosphorylated on tyrosine [29,33,34]. Also, addition of vanadate to intact cells has in many cases been shown to enhance the degree of phosphorylation of known targets of the tyrosine kinases. This is the case for the insulin receptor [17], $p60^{v\text{-}src}$ [18], the 36 kDa major cellular target for tyrosine phosphorylation [18], $p120^{v\text{-}abl}$, and several unidentified proteins in ANN-1 cells, a cell line transformed by Abelson leukemia virus (Klarlund and Forchhammer, unpublished results). For these reasons, we believe that treatment of cells with vanadate may be useful for the study and isolation of proteins that are phosphorylated on tyrosine at low levels in normal cells.

Transformation of cells by vanadate is presumably primarily mediated by inhibition of cellular phosphatases acting on phosphotyrosine [9–13]. In this study we found enhanced tyrosine kinase activities in extracts of cells treated with vanadate. The most obvious interpretation of these results is that cellular tyrosine kinases are activated in response to treatment with vanadate, which would be expected to contribute to elevate the content of phosphotyrosine. However, some caution is warranted in analyzing this type of measurement. The activities were measured in crude cell extracts, and it cannot be excluded that the observed enhanced angiotensin kinase activities could be due to modifications of unidentified modulatory molecules by the vanadate treatment. Also, only tyrosine kinases that are soluble in the extraction buffer were assayed, and the procedures involved in preparing the extracts may have resulted in alterations of the activities of the kinases. Finally, using angiotensin or any other substrate to monitor overall tyrosine kinase activities is complicated by the possibility that different tyrosine kinases may utilize the substrate with different efficiencies.

Activation of tyrosine kinase activities might occur by an indirect mechanism. For example, enhanced phosphorylation of certain tyrosine residues in the insulin receptor [15] and in the receptor for insulin-like growth factor I [16] activate the associated kinase activities. Vanadate may therefore activate cellular tyrosine kinases in an indirect fashion by inhibiting their dephosphorylation.

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