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Detection of Phosphotyrosine-Containing Proteins in Polyomavirus Middle Tumor Antigen-Transformed Cells after Treatment with a Phosphotyrosine Phosphatase Inhibitor

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Cells transformed with the middle tumor antigen (mT) of polyomavirus were treated with sodium orthovanadate (Na_3VO_4), an inhibitor of phosphotyrosine phosphatases, to enhance for the detection of cellular proteins which are phosphorylated on tyrosine. Na_3VO_4 treatment of mT-transformed rat F1-11 cells resulted in a 16-fold elevation in the level of phosphotyrosine associated with total cellular proteins. Parental F1-11 cells displayed only a twofold increase in phosphotyrosine following Na_3VO_4 treatment. The abundance of phosphotyrosine in Na_3VO_4-treated mT-transformed F1-11 cells was twofold higher than in untreated Rous sarcoma virus (RSV)-transformed F1-11 cells and 3.5-fold lower than in Na_3VO_4-treated RSV-transformed F1-11 cells. Tyrosine phosphorylation of many cellular proteins, including p36, the major substrate of the RSV $pp60^{v\text{-}src}$ protein, was detected in Na_3VO_4-treated mT-transformed F1-11 cells at levels comparable to those observed in RSV-transformed cells. Some of the major protein species recognized by antiphosphotyrosine antibodies in Na_3VO_4-treated mT-transformed cells displayed electrophoretic mobilities similar to those detected in RSV-transformed F1-11 cells. Tyrosine phosphorylation of p36 was also detected in fibroblasts infected with polyomavirus. There was no detectable difference in the kinase activity of $pp60^{c\text{-}src}$:mT extracted from untreated and Na_3VO_4-treated mT-transformed cells; however, Na_3VO_4 treatment of F1-11 and mT-transformed F1-11 cells was shown to inhibit the activity of phosphotyrosine phosphatases in a crude assay of total cellular activity with $pp60^{v\text{-}src}$ as the substrate. Thus, Na_3VO_4 treatment may allow the detection of phosphotyrosine-containing proteins in mT-transformed cells by preventing the turnover of phosphate on substrates phosphorylated by activated cellular protein-tyrosine kinases associated with mT. These results suggest that tyrosine phosphorylation of cellular proteins may be involved in the events that are responsible for mT-induced cellular transformation.

Oncogenic transformation of immortalized rodent cell lines by polyomavirus (PY) requires the expression of the middle tumor antigen (mT) (43, 47, 50). It has been demonstrated that mT is associated with $pp60^{c\text{-}src}$ (5, 16, 17), the cellular homolog of $pp60^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (RSV) (3, 4, 29). The association of mT with $pp60^{c\text{-}src}$ has been shown to cause an enhancement in the tyrosyl kinase activity of $pp60^{c\text{-}src}$, as detected by the phosphorylation of exogenous substrates in an immune complex kinase reaction (5, 9). While the nature of the activation of the mT-associated $pp60^{c\text{-}src}$ protein kinase activity is not known, characterization of these mT-complexed $pp60^{c\text{-}src}$ molecules has shown that they contain an altered site of tyrosine phosphorylation in vivo (10) and that they are phosphorylated on a novel tyrosine residue(s) within their amino-terminal domain in the immune complex kinase reaction in vitro (54).

The specific role of activated $pp60^{c\text{-}src}$ in transformation by PY has not been established. The analysis of mutant variants of mT has indicated that the ability of mT to activate $pp60^{c\text{-}src}$ may be necessary but not sufficient for mT-induced transformation; all transformation-competent forms of mT are capable of $pp60^{c\text{-}src}$ activation, but some mutants which induce partial transformation appear to fully activate $pp60^{c\text{-}src}$ protein kinase activity in vitro (5, 9, 17, 34). Recently Amini et al. (1) have examined the role of $pp60^{c\text{-}src}$ activation in mT transformation by suppressing $pp60^{c\text{-}src}$ synthesis through the expression of complementary antisense c-*src* RNA molecules in mT-transformed cells. Under conditions which resulted in an 8- to 10-fold reduction in the mT-activated $pp60^{c\text{-}src}$ protein kinase activity in vitro, the mT-transformed cells possessed a decreased capacity to form colonies in soft agar and foci on monolayers of untransformed cells as well as a diminished growth rate of tumors in vivo. Therefore, although the activation of $pp60^{c\text{-}src}$ may not be solely responsible for transformation by mT, these results support the contention that it is an important facet of mT function.

The evidence that mT activates the protein kinase activity of $pp60^{c\text{-}src}$ in vitro provides a possible mechanism for mT-induced transformation; however, there is no direct evidence that tyrosine phosphorylation of cellular proteins is involved in transformation by mT. PY mT transformation does not result in a detectable increase in total cellular phosphotyrosine (46), and no transformation-specific phosphotyrosine-containing cellular proteins have been identified in mT-transformed cells. In an attempt to enhance the detection of proteins phosphorylated on tyrosine in mT-transformed cells, we used sodium orthovanadate (Na_3VO_4), a potent inhibitor of purified cellular phosphotyrosine phosphatases (6, 23, 41). Na_3VO_4 treatment of cells has been shown to allow the detection of tyrosine phosphorylation of viral and cellular proteins that are not detected in untreated cells (7, 11, 48, 54). We have examined Na_3VO_4-

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treated mT-transformed and control cells and found a significant increase in the abundance of total cellular phosphotyrosine in mT-transformed cells. In addition, the 34,000- to 36,000-molecular-weight (34-36K) protein, a known phosphotyrosine-containing substrate in RSV-transformed cells, was shown to be phosphorylated on tyrosine in Na_3VO_4-treated PY mT-transformed and -infected cells. Finally, multiple species of phosphotyrosine-containing proteins were detected in Na_3VO_4-treated mT-transformed cell lysates by using a solid-phase immunoblot assay with anti-phosphotyrosine antibodies (anti-PTYR Abs).

MATERIALS AND METHODS

Cells and cell culture. The rat F1-11, FSV-2A2, and PY mT-transformed F2MT52 and F3MT51 cell lines (30) (the generous gift of Parmjit Jat, Massachusetts Institute of Technology) were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum. The rat RSV-transformed F1-11 (RSV-F1-11) and the mouse RSV-transformed 3T3 (RSV-3T3) cells were derived by transformation of F1-11 and NIH-3T3 cells, respectively, by RSV (Schmidt Ruppin D strain) and maintained in Dulbecco modified Eagle medium supplemented with 5% calf serum.

Virus infection. Primary mouse embryo fibroblasts (MEF) were prepared from 12- to 14-day-old embryos and infected with serial dilutions of an NG59RA stock, a wild-type PY revertant of the transformation-defective mutant NG59 (22), kindly provided by Tom Benjamin (Harvard Medical School).

Radiolabeling and sodium orthovanadate treatment of cells. Cells were labeled with $^{32}P_i$ (carrier-free; ICN, Irvine, Calif.) for 16 h in Dulbecco modified Eagle medium lacking phosphate and supplemented with 10% complete medium and 5% dialyzed calf serum. Cells were treated with 50 μM Na_3VO_4 (Sigma Chemical Co.) for 16 h in complete or phosphate-minus Dulbecco modified Eagle medium as indicated in the figure legends.

Phosphoamino acid analysis. Total cellular phosphoamino acid analysis was performed as described by Sefton et al. (46) except that all samples were lysed by boiling in 1% sodium dodecyl sulfate (SDS)–10 mM Tris (pH 7.2)–2 mM EDTA and then extracted immediately with phenol. Hydrolysis was performed in 6 N HCl (Pierce Chemical Co.) for 2 h at 110°C. Separation of the phosphoamino acids was performed by electrophoresis on 3MM paper (Whatman) at pH 3.5 in the first dimension for 2 h and electrophoresis at pH 1.9 in the second dimension for 4 h. The phosphoserine, phosphothreonine, and phosphotyrosine standards were detected by staining with ninhydrin. The radioactive phosphoamino acids were detected by autoradiography and quantitated by cutting out the phosphoamino acid standards and scintillation counting.

Antisera and antibodies. The mouse monoclonal antibody (MAb) 327, which recognizes $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$, was prepared as previously described (36). Rabbit antisera against p36 were kindly provided by Ray Erikson (20) (Harvard University) and Gerald Edelman (27) (Rockefeller University). Rabbit anti-PTYR Abs were purified on a phosphotyramine-Sepharose affinity column as described previously (51).

Immunoprecipitation and in vitro kinase reactions. Immunoprecipitations and in vitro kinase reactions were performed as described previously (36), except that the exogenous substrate enolase was used in the immune complex kinase reactions (5 μCi of [γ-^{32}P]ATP [carrier-free, ICN] and 1 μM unlabeled ATP per reaction). The immunoprecipitated proteins and in vitro kinase reaction products were analyzed by electrophoresis on 7.5 or 10% SDS-polyacrylamide gels (30:0.8, acrylamide-bisacrylamide) as noted. In the analysis of p36, the SDS-polyacrylamide gels were subjected to alkali treatment with 1 N KOH at 55°C for 1.5 h (13). All gels were dried, and autoradiography was performed with XAR-5 film (Kodak) and Cronex Lighting-Plus intensifying screens at −70°C for the time indicated in each figure legend.

Phosphotyrosine-specific phosphatase assays. Crude lysates from Na_3VO_4-treated and untreated cells were prepared by lysing the cells in buffer C (10 mM Tris [pH 7.2], 10 mM NaCl, 0.5 mM EDTA, 0.2% 2-β-mercaptoethanol) with 0.05% Nonidet P-40. The cell lysates were homogenized four to five times and clarified at 8,000 × *g* for 20 min at 4°C. Protein determination of the clarified lysate was performed by the method of Lowry et al. (37). Radiolabeled, phosphotyrosine-containing substrate for the phosphatase assay was prepared from unlabeled RSV-3T3 cell lysates by immunoprecipitation of $pp60^{src}$ and autophosphorylation in vitro with [γ-^{32}P]ATP as described above. The immune complexes were then washed once with RIPA buffer and twice with buffer C. The major radiolabeled products of the reaction are $pp60^{v\text{-}src}$ and a 130-kilodalton (kDa) protein, p130, both of which are phosphorylated only on tyrosine in vitro (L. A. Lipsich and J. S. Brugge, unpublished results). Various amounts of crude cell lysates were incubated with a constant amount of radiolabeled, phosphotyrosine-containing substrate at 30°C for 20 min. The reactions were stopped with the addition of 2× sample loading buffer and analyzed by electrophoresis on a 7.5% SDS-polyacrylamide gel. The gel was dried and exposed for autoradiography. The radioactive bands were excised and quantitated by scintillation counting.

Immunoblot analysis with anti-PTYR Abs. Lysates for immunoblot analysis were prepared from Na_3VO_4-treated and untreated cell lines by scraping cells into lysis buffer (2% SDS, 66 mM Tris [pH 6.8], 10 mM EDTA, and 0.3% 2-β-mercaptoethanol) and boiling for 10 min as described previously (51). Equal amounts of protein from cell lysates, determined by the method of Lowry et al. (37), were added to 2× sample loading buffer and subjected to electrophoresis on 10% SDS-polyacrylamide gels. The total cellular proteins were then electrophoretically transferred to nitrocellulose filters at 0.5 A for 2 h as described previously (49). The filters were incubated with a blocking buffer (5% bovine serum albumin [Pentex; Worthington Diagnostics], 50 mM Tris [pH 7.2], 150 mM NaCl) overnight at room temperature and subsequently probed with a 1:2,000 dilution (0.3 μg/ml) of anti-PTYR Abs in buffer (3% bovine serum albumin, 50 mM Tris [pH 7.2], 150 mM NaCl, 2% Nonidet P-40) for 1 h. The filters were washed three times and then incubated with ^{125}I-protein A (1 μCi/ml; Amersham Corp.) for 1 h. The filters were rewashed three times, dried, and subjected to autoradiography.

RESULTS

Sodium orthovanadate treatment of PY mT-transformed cells allows detection of increased levels of total cellular phosphotyrosine. To examine the effect of Na_3VO_4 treatment on the level of total cellular phosphotyrosine in PY mT-transformed cells, rat F2MT52 (F1-11 cells transformed with mT expressed on a pZipNeoSV(X)1 vector) and control, nontransformed FSV-2A2 (F1-11 cells containing only the

backbone pZipNeoSV(X)1 vector) (30) were labeled for 16 h with $^{32}P_i$ in the presence and absence of 50 μM Na_3VO_4. The cells were then harvested and assayed to examine the total cellular phosphoamino acid content (Fig. 1 and Table 1). RSV-F1-11 and the parental F1-11 cell lines were also included in this analysis. In the absence of Na_3VO_4 treatment, the levels of phosphotyrosine observed in the PY mT-transformed F2MT52 cells were comparable to those of the control FSV-2A2 and parental F1-11 cell lines. Following Na_3VO_4 treatment, there were increases in the percentage of total cellular phosphotyrosine as well as minor variations in the level of total cellular phosphoserine and phosphothreonine in each cell line analyzed. Both the FSV-2A2 and F1-11 cell lines showed only a twofold increase in total cellular phosphotyrosine after Na_3VO_4 treatment for 16 h. In contrast, the mT-transformed F2MT52 cells displayed a 15-fold increase in the level of phosphotyrosine (from 0.117 to 1.832% total cellular phosphotyrosine) following 16 h of Na_3VO_4 treatment. The increase in phosphotyrosine levels in F2MT52 cells was linear over the first 0 to 12 h of Na_3VO_4 treatment. Similar results were obtained from analysis of another PY mT-transformed F1-11 cell line (F3MT51) derived from an independent PY mT vector transfection (data not shown). These results provide evidence that Na_3VO_4 treatment of PY mT-transformed cells leads to a significant increase in total cellular phosphotyrosine compared with control, nontransformed cells and that the rise in the abundance of cellular phosphotyrosine in F2MT52 cells correlates with the expression of PY mT.

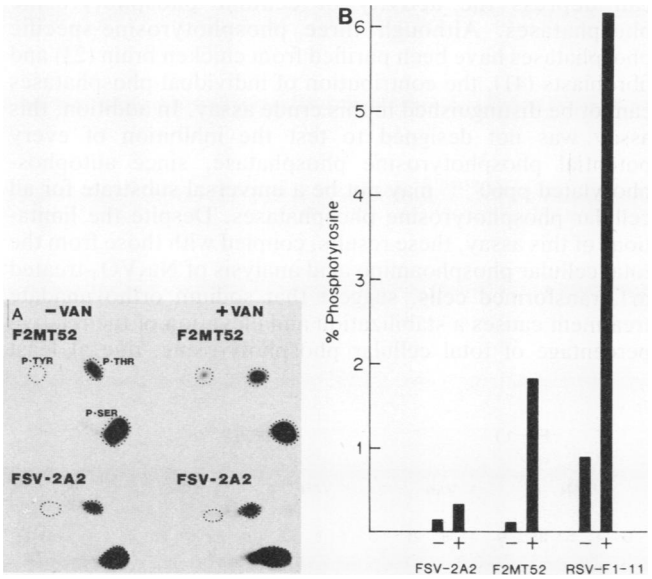

FIG. 1. Effect of sodium orthovanadate treatment on the phosphotyrosine content of FSV-2A2, F2MT52, and RSV-F1-11 cells. The cells were radiolabeled for 16 to 18 h with $^{32}P_i$ in the presence or absence of 50 μM Na_3VO_4 and processed as described in Materials and Methods. (A) Autoradiograms of the two-dimensional analysis of Na_3VO_4-treated and untreated mT-transformed F2MT52 cells (top two panels, exposed for 7 h at −70°C) and control FSV2A2 cells (lower two panels, exposed for 12 h at −70°C). The origin was in the upper left. Electrophoresis in the first dimension was from left (cathode) to right at pH 3.5 and in the second dimension from top (cathode) to bottom at pH 1.9. Only the region of the autoradiogram displaying the phosphoamino acids is shown. (B) Quantitation of the abundance of phosphotyrosine following treatment with and without Na_3VO_4. The radioactive spots corresponding to the ninhydrin-stained phosphoamino acid standards were excised from the paper and quantitated by scintillation counting. The height of the bars represents the percentage of total cellular phosphotyrosine present in Na_3VO_4-treated (+) and untreated (−) FSV-2A2, F2MT52, and RSV-F1-11 cell lines. The percentage of total cellular phosphotyrosine in Na_3VO_4-treated and untreated parental F1-11 cells was similar to the values found for FSV-2A2 (see Table 1).

TABLE 1. Relative abundance of acid-stable phosphoamino acids in sodium orthovanadate-treated and untreated control and mT- and RSV-transformed cell lines[a]

Cell line	Na_3VO_4 treatment	Phosphoamino acid content (% of total)			Increase in abundance of phosphotyrosine after Na_3VO_4 treatment (fold)
		Phosphotyrosine	Phosphothreonine	Phosphoserine	
F1-11	+	0.297	6.685	93.018	2.26
	−	0.131	5.298	94.571	
FSV2A2	+	0.306	6.154	93.539	1.95
	−	0.157	6.609	93.234	
F2MT52	+	1.832	9.274	88.894	15.66
	−	0.117	10.591	89.291	
RSV-F1-11	+	6.333	5.545	88.122	7.11
	−	0.891	7.498	91.611	

[a] Cells were labeled with $^{32}P_i$ for 16 h in the presence or absence of 50 μM Na_3VO_4, and total cellular phosphoamino acid analysis was performed as described in Materials and Methods.

The $pp60^{v\text{-}src}$-transformed RSV-F1-11 cells displayed a sevenfold increase in phosphotyrosine compared with control and mT-transformed cells in the absence of Na_3VO_4 treatment, similar to the analysis reported by Sefton et al. (46). Sodium orthovanadate treatment of RSV-F1-11 cells resulted in a further sevenfold increase in the abundance of phosphotyrosine, from 0.891 to 6.333% total cellular phosphotyrosine. The level of phosphotyrosine in RSV-F1-11 cells following Na_3VO_4 treatment was 21- and 3.5-fold greater than the levels of phosphotyrosine in Na_3VO_4-treated F1-11 and F2MT52 cells, respectively.

In vivo treatment with sodium orthovanadate inhibits cellular phosphotyrosine phosphatases. Two possible effects of sodium orthovanadate treatment in vivo that could account for the observed increases in total cellular phosphotyrosine in the F2MT52 and RSV-F1-11 cell lines are that (i) Na_3VO_4 treatment could stimulate the protein kinase activity of cellular or viral tyrosyl protein kinases and (ii) Na_3VO_4 treatment could inhibit cellular phosphotyrosine phosphatases, allowing the accumulation and detection of tyrosine phosphorylations on cellular proteins that normally turn over rapidly. There are several examples of tyrosyl protein kinases whose activity is enhanced following Na_3VO_4 treatment; both $pp60^{v\text{-}src}$ and the insulin receptor have previously been shown to possess enhanced protein kinase activity following Na_3VO_4 treatment (7, 11, 48). Lysis in the presence of Na_3VO_4 also has been reported to have an effect on the cellular *src* protein; however, in this case it appears that the preservation of tyrosine phosphorylation has an inhibitory effect on $pp60^{c\text{-}src}$ protein kinase activity (15). To determine whether orthovanadate treatment of F2MT52 cells in vivo affected the mT-associated $pp60^{c\text{-}src}$ protein kinase activity, F2MT52 and RSV-3T3 cells were treated with Na_3VO_4 for 16 h and lysed with RIPA buffer, and equal amounts of protein from the cell lysates were immunoprecipitated with either anti-$pp60^{src}$ MAb 327 or rabbit anti-mouse immunoglobulins. The protein kinase activity of the immuonprecipitated $pp60^{c\text{-}src}$ was examined with enolase as an exogenous substrate (Fig. 2). A small increase in the

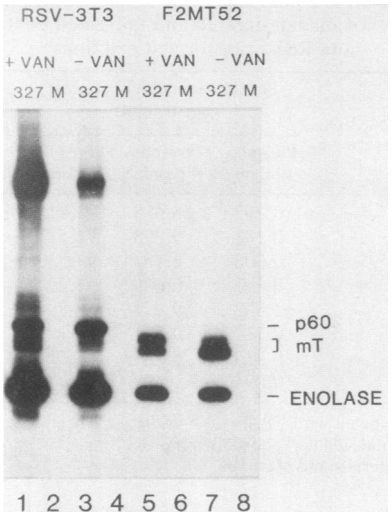

FIG. 2. Protein kinase activity of pp60^{src} from sodium orthovanadate (VAN)-treated and untreated RSV- and mT-transformed cells. RSV-3T3 (lanes 1 to 4) and F2MT52 (lanes 5 to 8) cells were treated with 50 μM Na_3VO_4 for 16 h (lanes 1, 2, 5, and 6) or untreated (lanes 3, 4, 7, and 8). Cell lysates were prepared and proteins were immunoprecipitated with MAb 327 (odd-numbered lanes) or rabbit anti-mouse immunoglobulin G (IgG) alone (even-numbered lanes). Immune complex kinase reactions were performed with the exogenous substrate enolase, as described in Materials and Methods. Autoradiograph exposure was for 60 h.

amount of enolase phosphorylation was observed in pp60$^{v\text{-}src}$ immunoprecipitates of RSV-3T3 cells treated with Na_3VO_4 compared with that in untreated RSV-3T3 cells (Fig. 2, lanes 1 and 3). This result is consistent with the findings of Collett et al. (11) and Brown and Gordon (7) that Na_3VO_4 treatment in vivo can stimulate the in vitro protein kinase activity of pp60$^{v\text{-}src}$. In contrast, no difference in the amount of enolase phosphorylation in vitro was observed in pp60$^{c\text{-}src}$ immunoprecipitates from Na_3VO_4-treated and untreated F2MT52 cells (Fig. 2, lanes 5 and 7). While these results provide evidence that the elevation in phosphotyrosine in sodium orthovanadate-treated F2MT52 cells is not due to a further enhancement in the mT-associated pp60$^{c\text{-}src}$ protein kinase activity, these results do not rule out that (i) Na_3VO_4 treatment could stimulate the activity of other cellular tyrosyl protein kinases in mT-transformed cells or (ii) Na_3VO_4 treatment could modulate the activity of serine- or threonine-specific protein kinases which might indirectly influence the activity of different cellular tyrosyl protein kinases.

To examine whether the Na_3VO_4-induced elevation in the level of total cellular phosphotyrosine in PY-transformed F2MT52 cells was accompanied by the inhibition of cellular phosphotyrosine phosphatases, we assayed the ability of crude cell lysates from treated and untreated cells to hydrolyze tyrosine phosphate with pp60$^{v\text{-}src}$ as the substrate. Although previous investigators have shown that Na_3VO_4 could inhibit purified phosphotyrosine-specific phosphatases in vitro (6, 23, 41), the effects of Na_3VO_4 treatment in vivo on cellular phosphotyrosine phosphatases had not been examined directly. Figure 3 shows the results of an assay of phosphotyrosine phosphatases in crude cell lysates from the control parental F1-11 and PY mT-transformed F2MT52 cell lines. Increasing amounts of lysate from untreated F1-11 (Fig. 3, lanes 1 to 4) and F2MT52 (lanes 9 to 12) cells displayed increasing levels of phosphatase activity as measured by the dephosphorylation of pp60$^{v\text{-}src}$ and p130, a phosphotyrosine-containing cellular protein which has been shown to coimmunoprecipitate with anti-pp60$^{v\text{-}src}$ antibody from RSV-transformed cells. Parallel experiments with a [^{35}S]methionine-labeled pp60$^{v\text{-}src}$ substrate showed little or no decrease in the amount of radiolabeled pp60$^{v\text{-}src}$ substrate from the same crude cell extracts containing up to 100 μg of protein (data not shown). These results suggest that the dephosphorylation of ^{32}P-labeled pp60$^{v\text{-}src}$ substrate is due to the action of phosphotyrosine phosphatases present in the crude cell lysates. There appeared to be more phosphotyrosine phosphatase activity per microgram of crude cell extract in the parental F1-11 cells than in the PY-transformed F2MT52 cells; however, this result varied slightly with each preparation of crude cell extracts.

Treatment of either F1-11 (Fig. 3, lanes 5 to 8) or F2MT52 (lanes 13 to 16) cells with Na_3VO_4 caused a significant reduction in the phosphatase activity detected in this assay. Dephosphorylation of pp60$^{v\text{-}src}$ was not detected in lysates containing less than 25 μg of cell protein. There was also a significant reduction in the dephosphorylation of p130. These results suggest that Na_3VO_4 treatment of nontransformed F1-11 and mT-transformed F2MT52 cell lines in vivo can depress the activity of cellular phosphotyrosine phosphatases. Although three phosphotyrosine-specific phosphatases have been purified from chicken brain (23) and fibroblasts (41), the contribution of individual phosphatases cannot be distinguished in this crude assay. In addition, this assay was not designed to test the inhibition of every potential phosphotyrosine phosphatase, since autophosphorylated pp60$^{v\text{-}src}$ may not be a universal substrate for all cellular phosphotyrosine phosphatases. Despite the limitations of this assay, these results, coupled with those from the total cellular phosphoamino acid analysis of Na_3VO_4-treated mT-transformed cells, suggest that sodium orthovanadate treatment causes a stabilization and elevation of the relative percentage of total cellular phosphotyrosine, due at least

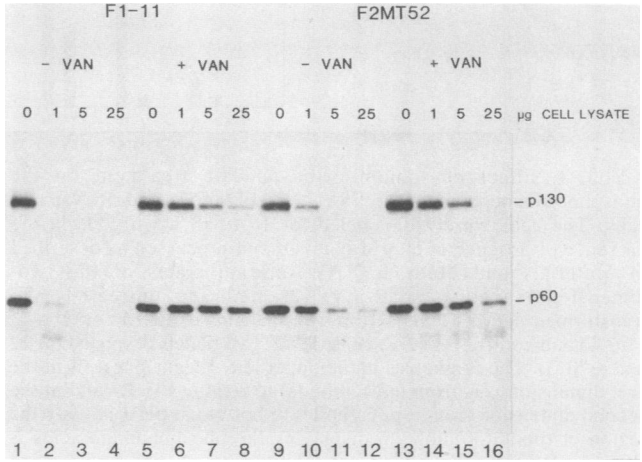

FIG. 3. Effect of sodium orthovanadate (VAN) treatment on phosphotyrosine-specific phosphatase activity from crude cell extracts. F1-11 (lanes 1 to 8) and F2MT52 (lanes 9 to 16) cells were treated with 50 μM Na_3VO_4 for 16 h (lanes 5 to 8 and 13 to 16) or untreated (lanes 1 to 4 and 9 to 12). The phosphotyrosine phosphatase activity from 0, 1, 5, and 25 μg of protein from cell lysates was assayed with ^{32}P-labeled pp60$^{v\text{-}src}$ and p130 as substrates as described in Materials and Methods. Autoradiograph exposure was for 16 h.

partly to the inhibition of phosphotyrosine-specific protein phosphatases within the cell.

p36 substrate of tyrosine protein kinases is phosphorylated in PY-transformed cells following treatment with sodium orthovanadate. Since the elevation of total cellular phosphotyrosine in F2MT52 cells following Na_3VO_4 treatment was consistent with the possibility that the activation of $pp60^{c\text{-}src}$ tyrosyl protein kinase activity in mT-transformed cells could result in an increase in tyrosine phosphorylation of cellular proteins, it was of interest to examine the phosphorylation of a known cellular substrate of the viral *src* protein in Na_3VO_4-treated mT-transformed cells. We chose to examine the 34-36K protein (p36), the most abundant phosphotyrosine-containing protein identified in RSV-transformed cells (21, 42). Tyrosine phosphorylation of p36 has never been reported in PY-transformed or -infected cells. To examine the phosphorylation of p36, parental F1-11, mT-transformed F2MT52, and $pp60^{v\text{-}src}$-transformed mouse RSV-3T3 cell lines were radiolabeled in vivo with $^{32}P_i$ in the presence (Fig. 4A, even-numbered lanes) or absence (Fig. 4A, odd-numbered lanes) of Na_3VO_4. The cells were harvested, lysed, and clarified in RIPA buffer, and immunoprecipitated with anti-p36 serum (Fig. 4A, lanes 1 to 8) or preimmune rabbit antiserum (Fig. 4A, lanes 9 to 16). Without treatment with Na_3VO_4, ^{32}P-labeled p36 was detectable only in the immunoprecipitates from RSV-3T3 cells (lane 7); however, following Na_3VO_4 treatment, p36 phosphorylation was detected in the mT-transformed F2MT52 and F3MT51, and $pp60^{v\text{-}src}$-transformed RSV-3T3 cells (lanes 4 and 8). No detectable p36 phosphorylation was observed in Na_3VO_4-treated or untreated parental F1-11 cells (lanes 1 and 2). Phosphoamino acid analysis of the radiolabeled p36 revealed mainly phosphotyrosine, with minor amounts of phosphoserine (data not shown). These results indicate that Na_3VO_4 treatment of $^{32}P_i$-labeled mT-transformed cells stabilizes the phosphorylation of p36, a known cellular phosphotyrosine substrate of $pp60^{v\text{-}src}$-transformed cells.

To further examine the mT specificity of p36 tyrosyl phosphorylation, we examined the phosphorylation of p36 in cells after infection with PY. It has previously been shown that the enhancement in the mT-associated $pp60^{c\text{-}src}$ protein kinase activity also occurs during productive infection with PY (5). MEF infected with different dilutions of a wild-type revertant PY stock, NG59RA (22), were radiolabeled with $^{32}P_i$, treated with 50 μM Na_3VO_4 for 12 h, and examined for phosphorylation of p36. Figure 4B shows the results of analysis of the anti-p36 immunoprecipitations from the Na_3VO_4-treated PY-infected MEF cells and mock-infected MEF cells. Phosphorylated p36 was readily detected in the PY-infected MEF cells treated with Na_3VO_4; the level of p36 phosphorylation correlated with the concentration of PY virus stock used for each infection (Fig. 4B, lanes 2, 4, 6, and 8). Very slight p36 phosphorylation was detected in the mock-infected MEF treated with Na_3VO_4 (Fig. 4B, lane 10). No phosphorylation of p36 was detected in PY-infected or mock-infected MEF in the absence of Na_3VO_4 treatment (data not shown). These results support the evidence from the experiment on the mT-transformed F2MT52 cells (Fig. 4A) indicating that expression of PY mT results in the tyrosine phosphorylation of a cellular protein, p36, whose phosphorylation is detectable only after treatment with Na_3VO_4.

Detection of phosphotyrosine-containing proteins in PY mT-transformed cells with anti-PTYR Abs. The phosphorylation of p36 in PY-transformed cells suggested that there may be similarities between the phosphotyrosine-containing

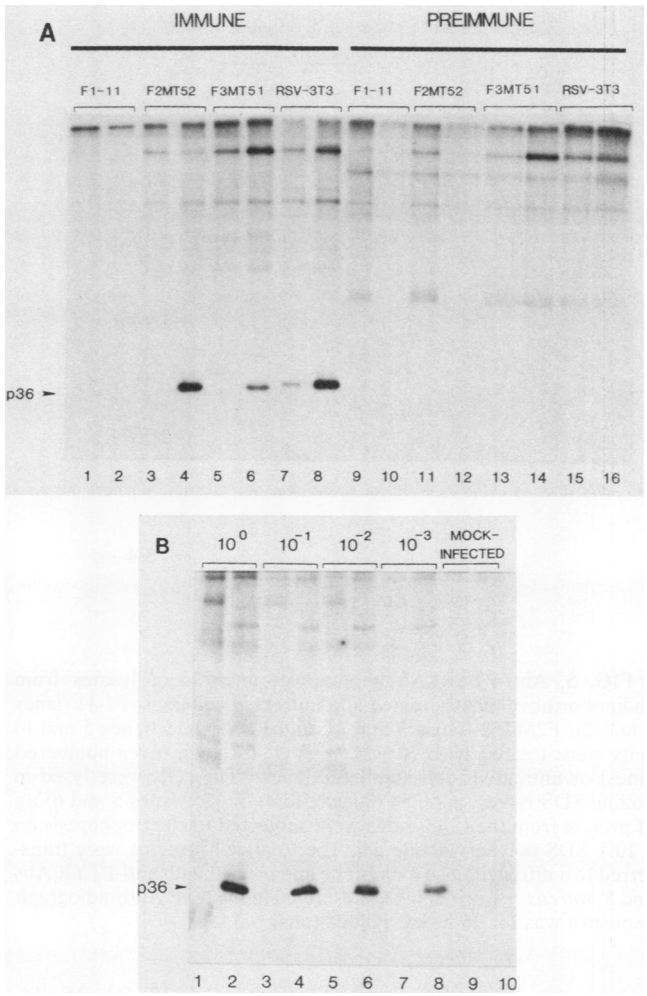

FIG. 4. Effect of sodium orthovanadate treatment on phosphorylation of p36 in mT-transformed and PY-infected cells. (A) Rat F1-11 (lanes 1, 2, 9, and 10), F2MT52 (lanes 3, 4, 11, and 12), and F3MT51 (lanes 5, 6, 13, and 14) and mouse RSV-3T3 (lanes 7, 8, 15, and 16) cells were radiolabeled in vivo with $^{32}P_i$ for 16 h in the presence (even-numbered lanes) and absence (odd-numbered lanes) of 50 μM Na_3VO_4. Cell lysates were prepared, and equal amounts of protein from cell lysates were immunoprecipitated with rabbit anti-p36 antiserum (lanes 1 to 8) or preimmune serum (lanes 9 to 16). The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels, followed by alkali treatment as described in Materials and Methods. Autoradiograph exposure was for 48 h. (B) Primary MEF that were infected with serial dilutions (10^0, 10^{-1}, 10^{-2}, and 10^{-3}) of a wild-type PY stock, NG59RA (lanes 1 to 8), or mock-infected (lanes 9 and 10) were radiolabeled with $^{32}P_i$ at 24 h postinfection for 16 h in the presence of 50 μM Na_3VO_4. The cells were harvested at 40 h postinfection, and cell lysates were prepared and immunoprecipitated with rabbit anti-p36 antiserum (even-numbered lanes) or preimmune serum (odd-numbered lanes). The immunoprecipitates were analyzed as for panel A. Autoradiograph exposure was for 72 h.

cellular substrates found in $pp60^{v\text{-}src}$-transformed cells and in Na_3VO_4-treated mT-transformed cells. To compare the phosphotyrosine-containing proteins from Na_3VO_4-treated and untreated F2MT52, RSV-F1-11, and F1-11 cells, these cell lines were subjected to solid-phase protein immunoblot analysis with anti-PTYR Abs. These antibodies have been useful in the identification and characterization of phosphotyrosine-containing substrates of several retrovirus transforming proteins and growth factor receptors which

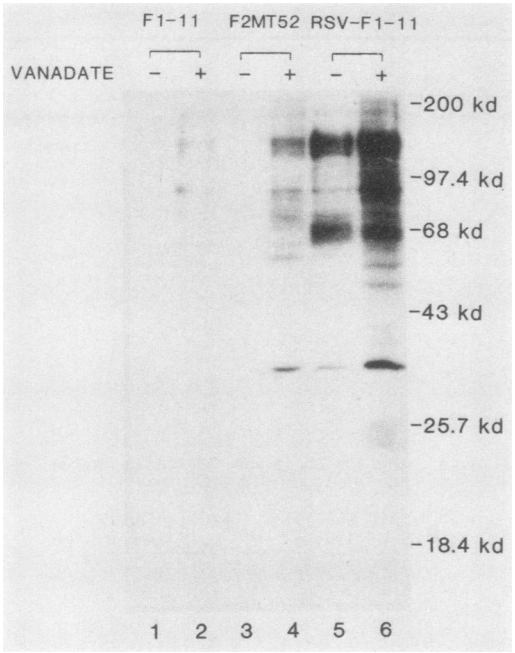

FIG. 5. Anti-PYTR Ab immunoblot analysis of lysates from sodium orthovanadate-treated and untreated cell lines. F1-11 (lanes 1 and 2), F2MT52 (lanes 3 and 4), and RSV-F1-11 (lanes 5 and 6) cells were treated with 50 μM Na_3VO_4 for 16 h (even-numbered lanes) or untreated (odd-numbered lanes). The cells were lysed in boiling SDS buffer, and 250 (lanes 1 to 4) or 125 (lanes 5 and 6) μg of protein from the cell lysates were subjected to electrophoresis on a 10% SDS-polyacrylamide gel. The total cell proteins were transferred to a nitrocellulose membrane and probed with anti-PTYR Abs and *S. aureus* ^{125}I-protein A as described in the text. Autoradiograph exposure was for 36 h. kd, Kilodaltons.

possess tyrosyl protein kinase activity (12, 19, 24, 35, 44, 51). Cell lysates from parental, mT-transformed, and RSV-transformed cell lines, treated with Na_3VO_4 and untreated, were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and probed with anti-PTYR Abs and ^{125}I-protein A from *Staphylococcus aureus* as described previously (Fig. 5). In the absence of Na_3VO_4 treatment, phosphotyrosine-containing cellular proteins were detected only in the RSV-F1-11 cells (Fig. 5, lane 5). No phosphotyrosine-containing proteins were detected in the untreated parental F1-11 or mT-transformed F2MT52 cell lines (Fig. 5, lanes 1 and 3).

Following Na_3VO_4 treatment, there were a few proteins of approximately 170, 130, and 80 kDa which were detected in the lysates of all three cell lines examined (Fig. 5, lanes 2, 4, and 6). These protein bands were very faint in the immunoblot analysis of parental F1-11 cells and much more prominent in the analysis of F2MT52 and RSV-F1-11 cells. This increase in intensity of the 170-, 130-, and 80-kDa bands in Na_3VO_4-treated mT- and RSV-transformed cells could be due to an increase in the tyrosine phosphorylation of these proteins as well as to tyrosine phosphorylation of other cellular proteins with similar molecular weights. There were a number of other immunoreactive proteins found specifically in lysates from both the F2MT52 and RSV-F1-11 cell lines following Na_3VO_4 treatment (Fig. 5, lanes 4 and 6). The profile of phosphotyrosine-containing proteins detected in Na_3VO_4-treated F2MT52 cells resembled that in untreated RSV-F1-11 cells both quantitatively and qualitatively; autoradiographic bands were detected in both mT- and RSV-transformed cells representing proteins of approximately 210, 116, 90, 72, and 32 kDa. There were also prominent immunoreactive proteins of approximately 65, 55, 48, 25, and 23 kDa observed exclusively in lysates of RSV-transformed cells.

Other investigators had previously found the majority of phosphotyrosine-containing proteins in RSV-transformed cells in the 30-kDa to 68-kDa size range (2, 39). Our results with the anti-PTYR Ab immunoblot analysis primarily detected larger phosphotyrosine-containing proteins, from 68 up to 210 kDa. These results suggest that the affinity of the anti-PTYR Abs used in this study may vary for different phosphotyrosine-containing substrates, as reported previously (51). Although it appears that the anti-PTYR Ab immunoblot analysis of Na_3VO_4-treated F2MT52 cells does not detect every phosphotyrosine-containing protein with equal efficiency, these results corroborate those obtained from the phosphoamino acid analysis and the examination of p36 phosphorylation, indicating that there is an increase in phosphotyrosine-containing proteins in mT-transformed cells and that the detection of these substrates requires the use of Na_3VO_4, a phosphotyrosine phosphatase inhibitor.

DISCUSSION

In this report we have attempted to determine whether the activated form of $pp60^{c\text{-}src}$ that is associated with PY mT causes a detectable increase in phosphotyrosine-containing cellular proteins following treatment with Na_3VO_4, an inhibitor of phosphotyrosine phosphatases. We have shown that there is a marked increase in the level of phosphotyrosine-containing proteins following Na_3VO_4 treatment in mT-transformed cells compared with treated untransformed control cells. Three lines of evidence support this conclusion: first, there was an eightfold-higher elevation in the level of total cellular phosphotyrosine following Na_3VO_4 treatment in mT-transformed cells than in control cells (Table 1); second, p36, a known cellular substrate of $pp60^{v\text{-}src}$, was significantly phosphorylated on tyrosine in Na_3VO_4-treated mT-transformed cells and was not phosphorylated in treated control cells (Fig. 4A); third, multiple phosphotyrosine-containing proteins were detected in the anti-PTYR Ab immunoblot analysis of lysates of Na_3VO_4-treated mT-transformed cells, while no proteins were detected in the absence of Na_3VO_4 treatment (Fig. 5).

The Na_3VO_4-dependent increase in the abundance of phosphotyrosine-containing proteins specifically correlated with the expression of mT and did not appear to represent a unique response of F2MT52 cells to Na_3VO_4 treatment. Na_3VO_4 treatment of an independently selected mT-transformed F1-11-derived cell line, F3MT51, also resulted in significant increases in the abundance of total cellular phosphotyrosine and in the level of phosphotyrosine-containing proteins, similar to those detected in F2MT52 cells (unpublished results). Furthermore, the procedures involved in transfection and neomycin treatment of the F2MT52 cell line did not select for cells that displayed a similar Na_3VO_4-dependent increase in the abundance of phosphotyrosine since FSV-2A2 cells, which carry the mT-minus pZipNeoSV(X)1 vector and were subjected to the same selection protocol, responded to Na_3VO_4 treatment in the same way as the parental F1-11 cell line. In addition, the evidence that PY infection of primary MEF resulted in significant phosphorylation of p36 following treatment with Na_3VO_4 further supports the mT specificity of the Na_3VO_4-dependent elevation of phosphotyrosine-containing proteins.

The increases in the abundance of phosphotyrosine observed following sodium orthovanadate treatment could be due to either Na_3VO_4-induced activation of tyrosyl protein kinases or inhibition of cellular phosphotyrosine-specific phosphatases or both. Na_3VO_4 treatment of mT-transformed cells did not appear to stimulate the protein kinase activity of the mT-associated $pp60^{c\text{-}src}$ molecules, as assayed by the phosphorylation of the exogenous substrate enolase. Although we cannot rule out the effect of Na_3VO_4 treatment on other cellular protein-tyrosine kinases, the evidence presented in this report that Na_3VO_4 treatment of cells causes an inhibition of phosphotyrosine phosphatases suggests that this inhibition could be responsible for the increases in the level of cellular phosphotyrosine by stabilizing tyrosine phosphorylations that normally turn over rapidly in vivo.

The identification of cellular proteins phosphorylated on tyrosine is an important step in defining the function of $pp60^{c\text{-}src}$ in transformation by mT. We have been able to detect the tyrosine phosphorylation of p36 and many other uncharacterized cellular proteins following Na_3VO_4 treatment. p36 is an abundant protein of the submembranous cortical cytoskeleton (14, 53) that is able to bind phospholipid, actin, and spectrin in a Ca^{2+}-dependent manner (25, 26, 31). Recently it has been proposed that p36 is a member of the family of lipocortinlike molecules that inhibit phosphorylase A_2 activity in vitro (8, 28, 45, 52). Despite this extensive characterization, the exact role of p36 in cell function and transformation is still unclear; examination of the effect of tyrosine phosphorylation on the activities of p36 should prove useful in determining its function. Although the other phosphotyrosine-containing proteins detected in the immunoblot analysis from Na_3VO_4-treated mT-transformed cells have not been rigorously characterized, many of these protein bands displayed electrophoretic mobilities similar to those of proteins identified in RSV-transformed cells. This result suggested that the phosphotyrosine-containing substrates detected in mT-transformed cells following Na_3VO_4 treatment may be a subset of those previously identified in $pp60^{v\text{-}src}$-transformed cells. Immunoprecipitation with anti-PTYR Abs and peptide mapping of the phosphotyrosine-containing proteins from Na_3VO_4-treated mT- and RSV-transformed cells will allow a more direct comparison of these putative cellular substrates.

In summary, the results presented in this report show a strong correlation between the expression of mT and an increase in the abundance of total cellular phosphotyrosine following Na_3VO_4 treatment. Recently it has been found that $pp61^{c\text{-}yes}$, the cellular homolog of the transforming protein of Y-73 sarcoma virus (4), interacts with mT in PY-transformed cells, in a unique complex analogous to the $pp60^{c\text{-}src}$:mT complex (S. Kornbluth, M. Sudol, and H. Hanafusa, unpublished results). It is currently not known how many different cellular protein-tyrosine kinases interact with mT or how each of these mT-associated protein-tyrosine kinases contributes to the elevation in total cellular phosphotyrosine reported here. Nevertheless, it is tempting to speculate from the results presented in this paper that the activation of tyrosyl protein kinase activity by mT is intimately involved in the elevation of phosphotyrosine-containing proteins in Na_3VO_4-treated mT-transformed cells and that this activation therefore plays an essential role in mT-induced transformation.

We analyzed the abundance of phosphotyrosine in other PY- and mT-transformed cell lines following treatment with Na_3VO_4. In all cases, there was an increase in the level of phosphotyrosine following Na_3VO_4 treatment that was similar to or greater than the increase reported here for F2MT52 cells. However, some of the control and parental cells of these other mT-transformed cell lines also displayed significant increases in the abundance of phosphotyrosine. Previously Klarlund (35) reported that growth of normal rat kidney cells in the presence of Na_3VO_4 also causes an elevation in the level of phosphotyrosine and, in addition, the acquisition of some phenotypes of transformation. Since little is known about the distribution and activity of cellular protein-tyrosine kinases and phosphotyrosine phosphatases in different cell lines, it is difficult to predict the effect of Na_3VO_4 treatment on the level of total cellular phosphotyrosine and the growth properties of cells. Variations in the abundance of phosphotyrosine-containing proteins following Na_3VO_4 treatment could reflect differences in the composition of cellular protein kinases and phosphatases or in the modulation of the activity of these enzymes by Na_3VO_4. These findings stress the importance of the selection of cell lines for analysis with Na_3VO_4. The control cell line must display a relatively small increase in the level of phosphotyrosine in response to Na_3VO_4 treatment for the effect of a protein tyrosine kinase on the abundance of phosphotyrosine to be assessed. The control FSV 2A2 and parental F1-11 cell lines provided a useful system for analysis of the effect of transformation by mT on tyrosine phosphorylation, since they exhibited a comparatively small increase in phosphotyrosine levels following Na_3VO_4 treatment.

Analysis of the $pp60^{v\text{-}src}$-transformed RSV-F1-11 cell line following Na_3VO_4 treatment indicated that there was also a significant increase in the level of total cellular phosphotyrosine compared with untreated cells. The anti-PTYR Ab immunoblot analysis indicated that there was an increase both in the number of phosphotyrosine-containing proteins and in the level of phosphorylation of substrates detected in Na_3VO_4-treated and untreated cells. Although many phosphotyrosine-containing proteins have been identified in RSV-transformed cells in the absence of Na_3VO_4 treatment, there is no direct genetic or physiological evidence correlating tyrosine phosphorylation of these substrates with any phenotypic changes associated with oncogenic transformation (14, 53). Analysis of a transformation-defective mutant of $pp60^{v\text{-}src}$ (18, 32) which retains full protein kinase activity has shown that there was no qualitative difference in the tyrosine phosphorylation of the eight most prominent substrates previously identified in RSV-transformed cells, although there were minor quantitative differences in the phosphorylation of some of the substrates (33). It is possible that the $pp60^{v\text{-}src}$-mediated phosphorylation of many of these putative substrates represents incidental phosphorylation events and that the cellular substrates relevant to transformation have yet to be discovered.

The results presented here raise the question of whether treatment with sodium orthovanadate can be useful in identification of the physiologically important cellular substrates of tyrosine-specific protein kinases. Unfortunately, no simple conclusion can be drawn from the experiments presented in this report. If treatment with a protein phosphatase inhibitor, such as Na_3VO_4, increases the background signal of phosphotyrosine-containing proteins which are inconsequential to transformation, then Na_3VO_4 treatment could further complicate the search for and identification of the crucial substrates of tyrosyl protein kinase-induced transformation. Alternatively, Na_3VO_4 treatment might be useful in the identification of substrates of protein tyrosine kinases which have so far escaped detection. Sodium orthovanadate

inhibition of phosphotyrosine phosphatases could aid in the detection of rare cellular phosphoproteins or of proteins on which the tyrosine phosphate turns over rapidly, by allowing the stabilization and accumulation of phosphotyrosine-containing cellular proteins in vivo. The experiments presented here indicate that the use of phosphatase inhibitors may be the only technique currently available to aid in the detection of phosphotyrosine-containing substrates involved in mT transformation. Indeed, while the mechanism of sodium orthovanadate action and its effects on cell growth and metabolism in vivo are complex (38, 40), Na_3VO_4 should prove to be a powerful tool to those searching for cellular substrates of tyrosyl protein kinases.

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LITERATURE CITED

1. **Amini, S., V. DeSeau, S. Reddy, D. Shalloway, and J. B. Bolen.** 1986. Regulation of $pp60^{c\text{-}src}$ synthesis in inducible RNA complementary to *c-src* mRNA in polyomavirus-transformed rat cells. Mol. Cell. Biol. **6**:2305–2316.
2. **Beemon, K., T. Ryden, and E. A. McNelly.** 1982. Transformation by avian sarcoma viruses leads to phosphorylation of multiple cellular proteins on tyrosine residues. J. Virol. **42**:742–747.
3. **Bishop, J. M.** 1982. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. **52**:301–354.
4. **Bishop, J. M., and H. Varmus.** 1982. Function and origins of retroviral transforming genes, p. 999–1108. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
5. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular *src* gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38**:767–777.
6. **Brautigan, D. L., P. Bornstein, and B. Gallis.** 1981. Phosphotyrosyl-protein phosphatase. J. Biol. Chem. **256**:6519–6522.
7. **Brown, D. J., and J. A. Gordon.** 1984. The stimulation of $pp60^{v\text{-}src}$ kinase activity by vanadate in intact cells accompanies a new phosphorylation state of the enzyme. J. Biol. Chem. **259**:9580–9586.
8. **Brugge, J. S.** 1986. The p35/p36 substrates of protein-tyrosine kinases as inhibitors of phospholipase A_2. Cell **46**:149–150.
9. **Cartwright, C. A., M. A. Hutchinson, and W. Eckhart.** 1985. Structural and functional modification of $pp60^{c\text{-}src}$ associated with polyomavirus middle tumor antigen from infected or transformed cells. Mol. Cell. Biol. **5**:2647–2652.
10. **Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart.** 1986. Altered site of tyrosine phosphorylation of $pp60^{c\text{-}src}$ associated with polyomavirus middle tumor antigen. Mol. Cell. Biol. **6**:1562–1570.
11. **Collett, M. S., S. K. Belzer, and A. F. Purchio.** 1984. Structurally and functionally modified forms of $pp60^{v\text{-}src}$ in Rous sarcoma virus-transformed cell lysates. Mol. Cell Biol. **4**:1213–1220.
12. **Comoglio, P. M., M. F. DiRenzo, G. Tarome, F. G. Giancotti, L. Naldini, and P. C. Marchisio.** 1984. Detection of phosphotyrosine-containing proteins in the detergent insoluble fraction of RSV-transformed fibroblasts by azobenzene phosphonate antibodies. EMBO J. **3**:483–489.
13. **Cooper, J. A., and T. Hunter.** 1981. Changes in protein phosphorylation of Rous sarcoma virus-transformed chicken embryo cells. Mol. Cell. Biol. **1**:165–178.
14. **Cooper, J. A., and T. Hunter.** 1983. Regulation of cell growth and transformation by tyrosine-specific protein kinases: the search for important cellular substrate proteins. Curr. Top. Microbiol. Immunol. **107**:125–161.
15. **Courtneidge, S.** 1985. Activation of the $pp60^{c\text{-}src}$ kinase by middle T binding or by dephosphorylation. EMBO J. **4**: 1471–1477.
16. **Courtneidge, S., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the *c-src* cellular gene. Nature (London) **303**:435–439.
17. **Courtneidge, S., and A. E. Smith.** 1984. The complex of polyoma virus middle-T antigen and $pp60^{c\text{-}src}$. EMBO J. **3**:585–591.
18. **Cross, F. R., E. A. Garber, D. Pellman, and H. Hanafusa.** 1984. A short sequence in the $p60^{src}$ N terminus is required for $p60^{src}$ myristylation and membrane association and for cell transformation. Mol. Cell. Biol. **4**:1834–1842.
19. **Ek, B., and C.-H. Heldin.** 1984. Use of an antiserum against phosphotyrosine for the identification of phosphorylated components in human fibroblasts stimulated by platelet-derived growth factor. J. Biol. Chem. **259**:11145–11152.
20. **Erikson, E., R. Cook, G. J. Miller, and R. L. Erikson.** 1981. The same normal cell protein is phosphorylated after transformation by avian sarcoma viruses with unrelated transforming genes. Mol. Cell. Biol. **1**:43–50.
21. **Erikson, E., and R. L. Erikson.** 1980. Identification of a cellular protein substrate phosphorylated by the avian sarcoma virus-transforming gene product. Cell **21**:829–836.
22. **Feunteun, J., L. Sompayrac, M. Fluck, and T. Benjamin.** 1976. Localization of gene functions in polyoma virus DNA. Proc. Natl. Acad. Sci. USA **73**:4169–4173.
23. **Foulkes, J. G., E. Erikson, and R. L. Erikson.** 1983. Separation of multiple phosphotyrosyl- and phosphoseryl-protein phosphatases from chicken brain. J. Biol. Chem. **258**:431–438.
24. **Frackelton, A. R., Jr., A. H. Ross, and H. N. Eisen.** 1983. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus-transformed cells and growth factor-stimulated cells. Mol. Cell. Biol. **3**:1343–1352.
25. **Gerke, V., and K. Weber.** 1984. Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders: calcium dependent binding to non-erythroid spectrin and F-actin. EMBO J. **3**:227–233.
26. **Glenney, J. R., Jr.** 1986. Phospholipid dependent Ca^{2+}-binding by the 36Kd tyrosine kinase substrate (calpactins) and its 33Kd core. J. Biol. Chem. **261**:7247–7252.
27. **Greenberg, M. J., R. Brackenbury, and G. Edelman.** 1984. Changes in the distribution of the 34-kdalton tyrosine kinase substrate during differentiation and maturation of chicken tissues. J. Biol. Chem. **98**:473–486.
28. **Huang, K.-S., B. P. Wallner, R. J. Mattaliano, R. Tizard, C. Burne, A. Frey, C. Hession, P. McGray, L. K. Sinclair, E. P. Chow, J. L. Browning, K. L. Ramachandran, J. Tang, J. E. Smart, and R. B. Pepinsky.** 1986. Two human 35 kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. Cell **46**:191–199.
29. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
30. **Jat, P. S., C. L. Cepko, R. C. Mulligan, and P. A. Sharp.** 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. Mol. Cell. Biol. **6**:1204–1217.
31. **Johnsson, N., J. Vandekerckhove, J. Van Damme, and K. Weber.** 1986. Binding sites for calcium, lipid and p11 on p36, the substrate of retroviral tyrosine-specific protein kinases. FEBS Lett. **198**:361–364.
32. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1985. Mutation of the NH_2-terminal glycine of $pp60^{src}$ prevents fatty acylation and morphological transformation. Proc. Natl. Acad. Sci. USA **82**:4625–4628.
33. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1986. Rous sarcoma

virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation. Cell **45**:105–112.

34. **Kaplan, D. R., M. Whitman, B. Schaffhausen, L. Raptis, R. L. Garcea, D. Pallas, T. M. Roberts, and L. Cantley.** 1986. Phosphatidylinositol metabolism and polyoma-mediated transformation. Proc. Natl. Acad. Sci. USA **83**:3624–3628.
35. **Klarlund, J. K.** 1985. Transformation of cells by an inhibitor of phosphatases acting on phosphotyrosine in proteins. Cell **41**:707–717.
36. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies which recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
37. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
38. **Macara, I. G.** 1980. Vanadium—an element in search of a role. Trends Biochem. Sci. **5**:92–94.
39. **Martinez, R., K. D. Nakamura, and M. J. Weber.** 1982. Identification of phosphotyrosine-containing proteins in untransformed and Rous sarcoma virus-transformed chicken embryo fibroblasts. Mol. Cell. Biol. **2**:653–665.
40. **Nechay, B. R.** 1984. Mechanisms of action of vanadium. Annu. Rev. Pharmacol. Toxicol. **24**:501–524.
41. **Nelson, R. L., and P. E. Branton.** 1984. Identification, purification, and characterization of phosphotyrosine-specific protein phosphatases from cultured chicken embryo fibroblasts. Mol. Cell. Biol. **4**:1003–1012.
42. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific protein phosphorylation contains phosphotyrosine. Cell **21**:821–828.
43. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
44. **Ross, A. H., D. Baltimore, and H. N. Eisen.** 1981. Phosphotyrosine containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten. Nature (London) **294**:654–656.
45. **Saris, C. J. M., B. F. Tack, T. Kristensen, J. R. Glenney, Jr., and T. Hunter.** 1986. The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell **46**:201–212.
46. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
47. **Smith, A. E., and B. K. Ely.** 1983. The biochemical basis of transformation by polyoma virus. Adv. Viral Oncol. **3**:3–30.
48. **Tamura, S., T. A. Brown, J. H. Whipple, Y. Fujita-Yamaguchi, R. E. Dubler, K. Cheng, and J. Larner.** 1984. A novel mechanism for the insulin-like effect of vanadate on glycogen synthase in rat adipocytes. J. Biol. Chem. **259**:6650–6658.
49. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
50. **Treisman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature (London) **292**:595–600.
51. **Wang, J. Y. J.** 1985. Isolation of antibodies for phosphotyrosine by immunization with a v-*abl* oncogene-encoded protein. Mol. Cell. Biol. **5**:3640–3643.
52. **Weber, K., and N. Johnsson.** 1986. Repeating sequence homologies in the p36 target proteins of retroviral protein kinases and lipocortin, the p37 inhibitor of phospholipase A_2. FEBS Lett. **203**:95–98.
53. **Weber, M. J.** 1984. Malignant transformation by Rous sarcoma virus: from phosphorylation to phenotype. Adv. Viral Oncol. **4**:249–268.
54. **Yonemoto, W., M. Jarvis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel.** 1985. Novel tyrosine phosphorylation within the amino terminal domain of $pp60^{c\text{-}src}$ molecules associated with polyoma virus middle tumor antigen. Proc. Natl. Acad. Sci. USA **82**:4568–4572.