MOLECULAR AND CELLULAR BIOLOGY, May 1985, p. 916–922
0270-7306/85/050916-07$02.00/0

Vol. 5, No. 5

Characterization of pp60^{src} Phosphorylation In Vitro in Rous Sarcoma Virus-Transformed Cell Membranes

MARILYN D. RESH* AND R. L. ERIKSON

Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138

Received 31 October 1984/Accepted 24 January 1985

Phosphorylation of the *src* gene product pp60$^{v\text{-}src}$ was studied in plasma membrane fractions prepared from Rous sarcoma virus-transformed vole cells. Upon addition of [γ-^{32}P]ATP to isolated membrane vesicles, phosphate was incorporated into a 60,000-dalton polypeptide identified as pp60$^{v\text{-}src}$. In the presence of vanadate, pp60$^{v\text{-}src}$ phosphorylation was stimulated ca. 30-fold. At low concentrations of ATP (1 μM), this reaction occurred almost exclusively on the carboxy-terminal 26,000-dalton region of pp60$^{v\text{-}src}$. However, at higher ATP concentrations (100 μM), additional sites of phosphorylation were evident in the amino-terminal 34,000-dalton region. Kinetic analyses, performed under conditions in which ATP hydrolysis was minimal, revealed that the phosphorylation reaction at the carboxy terminus exhibited a higher V_{max} and a lower K_m for ATP than those occurring at the amino terminus. In addition, the amino-terminal region of pp60$^{v\text{-}src}$ was more rapidly dephosphorylated than the carboxy-terminal region. These results indicate that interaction of pp60$^{v\text{-}src}$ with the plasma membrane may limit the extent of amino-terminal phosphorylation by lowering the rate of the reaction and the affinity for the substrate while increasing its susceptibility to phosphoprotein phosphatases. We suggest that the use of transformed-cell membrane preparations provides a model system for studying the possible regulatory roles of phosphorylation and dephosphorylation on pp60$^{v\text{-}src}$ function.

Cellular transformation by Rous sarcoma virus (RSV) is mediated by the expression of the viral *src* gene product pp60$^{v\text{-}src}$ (21). This 60,000-dalton polypeptide (2, 14, 31, 32) exhibits protein kinase activity specific for tyrosine residues and is itself a phosphoprotein (7, 8, 15, 18, 22, 28, 29). Studies of pp60$^{v\text{-}src}$ immunoprecipitated from RSV-infected cells have identified a serine residue in amino-terminal region of the molecule (Ser-17) and a tyrosine residue in the carboxy-terminal domain (Tyr-416) as the major sites of phosphorylation (6, 36, 39). Phosphorylation of Ser-17 is mediated by a cyclic AMP-dependent protein kinase, whereas the carboxy-terminal tyrosine phosphorylation occurs through a cyclic AMP-independent reaction (6). The phosphorylation of Tyr-416 probably represents pp60$^{v\text{-}src}$ autophophorylation, since it occurs when purified preparations of pp60$^{v\text{-}src}$ are phosphorylated in vitro (20, 30). Interestingly, upon addition of increasing concentrations of ATP, additional sites of tyrosine phosphorylation in the amino-terminal region of pp60$^{v\text{-}src}$ have been observed (1, 9, 20). Since these sites had not been identified in in vivo-labeled cells, their physiological significance was unclear.

Biochemical and cytological investigations have established that a significant fraction of the pp60$^{v\text{-}src}$ in transformed cells is associated with the plasma membrane (11, 23, 25, 26, 33) through a domain near the amino terminus (12, 24). There is a strong correlation between membrane association of pp60$^{v\text{-}src}$ and expression of cellular transformation parameters (10, 17, 24). Initial studies by Garber et al. (17) demonstrated that addition of [γ-^{32}P]ATP to isolated plasma membranes from RSV-transformed cells results in phosphorylation of pp60$^{v\text{-}src}$ in the carboxy-terminal region of the molecule. These experiments were performed with ATP concentrations well below the K_m of the enzyme for ATP. Therefore, it was logical to question whether incubation with higher ATP concentrations would allow amino-terminal phosphorylation to occur in membrane-bound pp60$^{v\text{-}src}$ or whether interaction with the plasma membrane blocked the amino-terminal phosphorylation reaction. It was also not clear whether phosphorylation at the amino terminus represented a physiologically relevant reaction or was an artifact of performing kinase reactions in vitro.

To resolve these issues, we prepared plasma membrane-enriched fractions from RSV-transformed vole cells and established conditions which maximized phosphate incorporation into pp60$^{v\text{-}src}$. In this paper, we describe kinetic studies of phosphorylation of amino- and carboxy-terminal sites in membrane-bound pp60$^{v\text{-}src}$ and suggest that both kinetic parameters and the presence of phosphoprotein phosphatases may limit the extent of amino-terminal phosphorylation. While this work was in progress, Collett et al. (5) and Brown and Gordon (1) reported the identification of variant forms of pp60$^{v\text{-}src}$ containing amino-terminal phosphotyrosine in transformed cell lysates. Our work with membrane vesicles complements and extends these findings.

MATERIALS AND METHODS

Cells and virus. European field vole (*Microtus agrestis*) cells transformed by the Schmidt-Ruppin strain of RSV, subgroup D (clone 1T), were maintained in culture at 37°C.

Antisera. Antiserum specific for p60^{src} (αp60) was used as previously described (19). Preparation of antiserum specific for the carboxy-terminal 26,000-dalton region of p60 will be described in a future publication. This antiserum (αC) efficiently immunoprecipitated pp60$^{v\text{-}src}$ from in vivo-labeled cells. Under in vitro phosphorylation conditions, no pp60^{src} autophosphorylation was observed and addition of αC antiserum blocked the immunoglobulin G kinase reaction of tumor bearing rabbit antibody.

Membrane preparation. A plasma membrane-enriched membrane fraction was prepared as described previously (32a). Briefly, cell monolayers were rinsed twice with STE (150 mM NaCl, 50 mM Tris, 1 mM EDTA [pH 7.2]) and removed from the dish by vigorous pipetting with ice-cold STE. Cells were collected by low-speed centrifugation,

* Corresponding author.

allowed to swell in 0.8 ml of hypotonic buffer (10 mM Tris, 0.2 mM $MgCl_2$ [pH 7.4]) per 100-mm dish for 10 min at 0°C, and disrupted by 30 up-and-down strokes in a Dounce homogenizer with a tight-fitting pestle. The homogenate was adjusted to a final concentration of 0.25 M sucrose and 1 mM EDTA and centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was removed immediately and saved, and the pellet was suspended in 0.25 M sucrose–10 mM Tris–1 mM EDTA (pH 7.4) by 10 strokes in a Dounce homogenizer. After a 10-min centrifugation at 1,000 × *g*, the supernatant was removed, combined with the supernatant from the first 1,000 × *g* spin, and centrifuged at 100,000 × *g* for 1 h at 3°C in a type 65 rotor. The resultant pellet, denoted P100, was suspended in Tris-Mg^{2+} buffer (10 mM Tris, 5 mM $MgCl_2$ [pH 7.4]) by Dounce homogenization (ca. 1 to 2 mg of protein per ml), divided into portions, rapidly frozen in liquid nitrogen, and stored at −70°C.

Phosphorylation assays. Frozen 1T P100 membranes were thawed and diluted with 2 volumes of Tris-Mg^{2+} buffer. The reaction mixture contained 100 μl of membranes, 1 mM Na_3VO_4, and 0.1 to 200 μM ATP. In some experiments, the reaction was performed in a final volume of 1.0 ml to reduce the amount of extraneous ATP hydrolysis. Assays were initiated by the addition of 50 μCi of [γ-^{32}P]ATP, followed by incubation at 22°C for 1 min (to determine initial rates) or 15 min (steady state). The reaction was quenched by the addition of disodium EDTA to a concentration of 10 mM and adjusted to RIPA buffer (10 mM Tris, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate) at 4°C.

Immunoprecipitation and polyacrylamide gel analysis. Reaction mixtures in RIPA buffer were immunoprecipitated in a volume of 1.0 ml with 3 μl of αp60 antiserum (19), processed as previously described (2), and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27) on 10% polyacrylamide gels. Dried gels were exposed to either Kodak XAR or Cronex 4 X-ray film with Lightning-Plus intensifying screens (E. I. duPont de Nemours & Co., Inc., Wilmington, Del.) at −70°C. The amount of radiolabel incorporated into pp60^{src} was quantitated by excising the pp60 band from dried gels and counting it in 4 ml of toluene-based liquid scintillation fluid. For one-dimensional peptide mapping, the bands corresponding to pp60^{src} were excised from wet gels and treated with 20 ng of *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.). At this concentration, complete digestion of pp60^{src} into 34,000- and 26,000-dalton fragments was achieved, without significant cleavage of the 34,000-dalton fragment into smaller peptides. Reelectrophoresis through 10% polyacrylamide gels was performed as described by Cleveland et al. (4).

Assay of ATP hydrolysis. At various times during the phosphorylation reaction, 1-μl portions were spotted on polyethyleneimine cellulose thin-layer chromatography (Brinkmann Instruments, Inc., Westbury, N.Y.) and chromatographed in 0.4 M Na_2HPO_4 (pH 3.5). The spots corresponding to ATP and inorganic phosphate were localized by autoradiography, excised from the plate, and quantitated by liquid scintillation spectrometry.

RESULTS

To study phosphorylation of pp60$^{v\text{-}src}$ in vitro, a plasma membrane-enriched population (P100) was prepared from RSV-transformed vole cells. Incubation of this membrane fraction with [γ-^{32}P]ATP resulted in phosphate incorporation into numerous proteins, including a 60,000-dalton polypeptide. The latter was identified as pp60$^{v\text{-}src}$ based on specific immunoprecipitation by αp60 antibody (Fig. 1, lanes 1 and 2). Since the plasma membrane contains ATPases and phosphoprotein phosphatases which might restrict the extent of protein phosphorylation, we sought to establish conditions to maximize phosphate incorporation into pp60$^{v\text{-}src}$. When 1 mM Na_3VO_4, a potent ATPase and phosphatase inhibitor (3, 35, 38), was included in the reaction, a dramatic increase in the amount of ^{32}P incorporated into pp60$^{v\text{-}src}$ was observed (Fig. 1, lanes 3 and 4). Depending on experimental conditions, vanadate-treated membranes incorporated 5- to 30-fold more phosphate into pp60$^{v\text{-}src}$ than did untreated samples. The variation in the degree of vanadate stimulation seemed to be dependent on the ATP concentration in the assay. At low ATP concentrations (0.5 μM), greater stimulation of pp60^{src} phosphorylation was observed (15- to 30-fold, Table 1 and Fig. 1) than at higher ATP concentrations (100 μM; see Fig. 2). Other phosphatase inhibitors, such as fluoride and Zn^{2+}, were ineffective, whereas vanadyl ions were partially effective in stimulating pp60$^{v\text{-}src}$ phosphorylation when compared with the effect of vanadate (Table 1). The inclusion of KCl or NaCl in the presence of vanadate reduced the amount of pp60$^{v\text{-}src}$ phosphorylation when compared with vanadate alone. Soybean trypsin inhibitor, a compound reported to prevent proteolysis of pp60$^{v\text{-}src}$ in cell lysates (40), did not significantly affect the levels of phosphorylated pp60$^{v\text{-}src}$ that we detected (Table 1). However, antibodies directed against the carboxy-terminal region of pp60$^{v\text{-}src}$ (see above, the putative kinase domain, were highly effective inhibitors of the reaction (Table 1). This implies that the reaction we

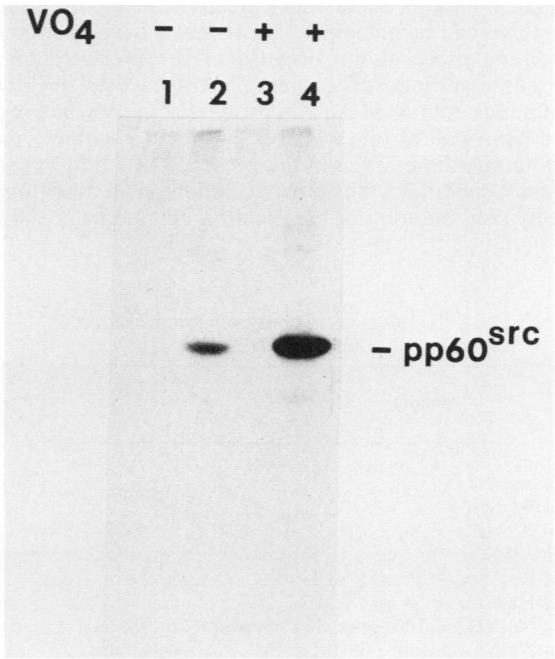

FIG. 1. Vanadate stimulation of membrane-bound pp60$^{v\text{-}src}$ phosphorylation in vitro. Membrane fractions in 100 μl were incubated with 50 μCi of [γ-^{32}P]ATP (0.5 μM) for 15 min at 22°C in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 1 mM vanadate. The reactions were quenched by the addition of EDTA, immunoprecipitated with nonimmune rabbit serum (lanes 1 and 3) or αp60 antiserum (lanes 2 and 4), and analyzed by polyacrylamide gel electrophoresis as described in the text.

monitored is pp60$^{v\text{-}src}$ autophosphorylation. Thus, we demonstrated that in the presence of Mg^{2+} and ATP, phosphorylation of membrane-bound pp60$^{v\text{-}src}$ can be detected and that this reaction is greatly enhanced by vanadate ions.

The dependence of pp60$^{v\text{-}src}$ phosphorylation levels on the concentration of vanadate was quantitated and is illustrated in Fig. 2. The maximal effect of vanadate was exerted at approximately 10 μM. All subsequent experiments were performed in the presence of 100 μM to 1 mM Na_3VO_4.

During the course of this investigation, it became apparent that incubation with increasing concentrations of ATP produced phosphorylated pp60$^{v\text{-}src}$ which migrated as a doublet band on polyacrylamide gels. Previous work on purified preparations of pp60$^{v\text{-}src}$ had established that this behavior was due to the appearance of additional tyrosine phosphorylation sites in the amino-terminal region of the molecule (9, 20). Accordingly, the location of the sites phosphorylated in membrane-bound pp60$^{v\text{-}src}$ was probed by one-dimensional peptide analysis. At ATP concentrations of 1 μM and lower, radiolabel was incorporated almost exclusively into the 26,000-dalton V8 fragment derived from the carboxy terminus (Fig. 3, lanes 1 and 2). As the ATP was increased to 100 μM, phosphorylation in the amino-terminal region occurred as well (Fig. 3, lanes 3 through 6). Phosphoamino acid analyses confirmed that all these reactions were on tyrosine residues. This implies that interaction with the plasma membrane does not block the accessibility of the amino-terminal sites of phosphorylation.

Since membrane-bound pp60$^{v\text{-}src}$ could be differentially phosphorylated depending on the ATP concentration, it was of interest to determine the kinetic parameters of this reaction. These analyses require the establishment of experimental conditions under which steady-state incorporation of phosphate can be achieved. This is not a trivial matter with membrane preparations because of the presence of ATP hydrolases and phosphoprotein phosphatases. The 1T P100 membranes contained ATP hydrolytic activity which hydrolyzed nearly all of the available [γ-^{32}P]ATP within 5 min at room temperature, even in the presence of 1 mM vanadate. The only conditions which proved effective in inhibiting this activity were dilution of the reaction volume from 100 μl to 1 ml and addition of carrier, nonradioactive ATP. When the phosphorylation reaction was performed under these conditions, steady-state incorporation of phosphate into pp60$^{v\text{-}src}$ was achieved in 15 min at 22°C (Fig. 4). Since the reaction was linear for at least 1 min, a time at which greater than 90% of the initial [γ-^{32}P]ATP remained unhydrolyzed, we were able to evaluate the initial rate of both amino- and carboxy-terminal phosphorylation reactions as a function of ATP concentration. The results of these analyses, which exhibited classical Michaelis-Menten kinetics, are illustrated in Fig. 5. The apparent K_m for ATP was approximately 3 μM (± 1 μM) for phosphorylation in the carboxy-terminal region, wheras amino-terminal phosphorylation exhibited an apparent K_m of 25 μM (± 6 μM) ATP (n = 3 experiments). Moreover, the carboxy-terminal V_{max} was two- to threefold higher than the V_{max} for the amino-terminal reaction (Fig. 5). Taken together, these data reveal that pp60$^{v\text{-}src}$-catalyzed phosphorylation of sites in the amino-terminal region of the molecule exhibits a lower affinity for substrate (ATP) and proceeds more slowly than the reaction at the carboxy terminus.

In general, membrane vesiculation often occurs after Dounce homogenization of many cell types. We believe this is also the case with the 1T P100 fractions, since addition of low concentrations of nonionic detergent, which did not release pp60$^{v\text{-}src}$ from the membrane, resulted in a twofold increase in the amount of phosphate incorporation. Since pp60$^{v\text{-}src}$ is exposed only on the inner face of the plasma membrane (41), in our system only the inside-out vesicles would support pp60$^{v\text{-}src}$ autophosphorylation. To ensure that we were not excluding the contribution of other populations of vesicles, we performed the experiment of Fig. 5 in the presence of 0.1% octyl glucoside. The K_m and V_{max} values

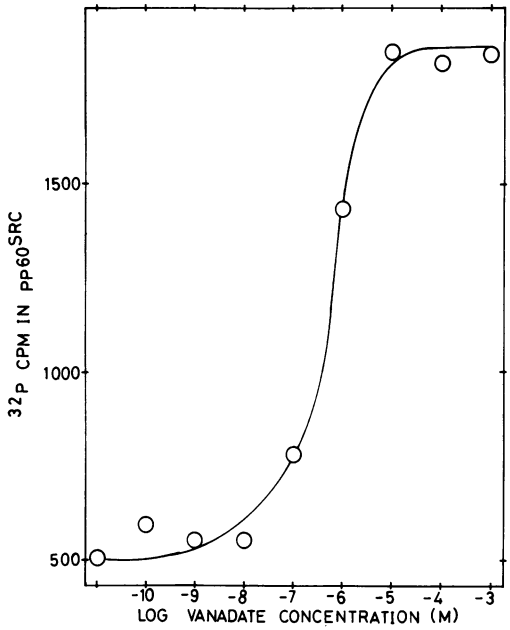

FIG. 2. Dependence of pp60$^{v\text{-}src}$ phosphorylation on vanadate concentration. Membrane fractions in 100 μl were incubated with 50 μCi of [γ-^{32}P]ATP (100 μM) for 15 min at 22°C in the presence of the indicated concentrations of vanadate (0 to 1 mM). The reactions were quenched and immunoprecipitated with αp60 antiserum, and the amount of ^{32}P incorporated into pp60$^{v\text{-}src}$ was determined after gel electrophoresis. Half-maximal stimulation was obtained at ca. 0.5 μM vanadate.

TABLE 1. Effect of various compounds on pp60$^{v\text{-}src}$ phosphorylation[a]

Addition	% of ^{32}P-labeled pp60$^{v\text{-}src}$
None	3
1 mM Na_3VO_4	100
1 mM $VOSO_4$	22
100 mM NaF	2
25 mM $ZnSO_4$	3
1 mM Na_3VO_4 + 50 mM KCl	72
1 mM Na_3VO_4 + 50 mM NaCl	45
1 mM Na_3VO_4 + 100 μg of STI[b] per ml	104
1 mM Na_3VO_4 + anti-p60 antibody[c]	18

[a] Membrane fractions in 100 μl were incubated with the indicated compounds and 50 μCi of [γ-32P]ATP (0.5 μM) for 15 min at 22°C. The reactions were quenched by the additon of EDTA, immunoprecipitated with αp60 serum, and analyzed by polyacrylamide gel electrophoresis as described in the text. The radiolabeled bands corresponding to pp60^{src} were excised from the dried gel, and the radioactivity incorporated was quantitated by liquid scintillation spectrometry.

[b] STI, Soybean trypsin inhibitor.

[c] Antibody specific for the carboxy-terminal 26,000-dalton V8 fragment of pp60$^{v\text{-}src}$.

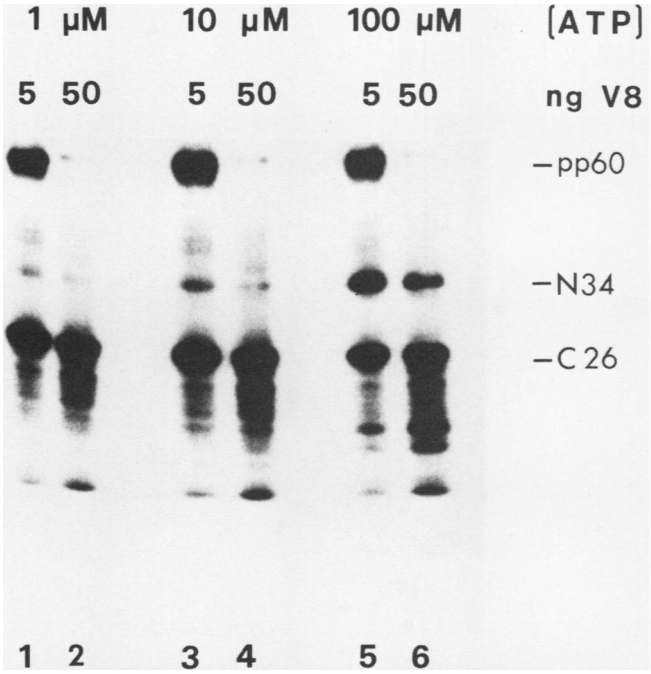

FIG. 3. One-dimensional V8 proteolytic maps of membrane-bound phosphorylated pp60$^{v\text{-}src}$. Membrane fractions phosphorylated in the presence of 1 mM vanadate and 1 (lanes 1 and 2), 10 (lanes 3 and 4), or 100 (lanes 5 and 6) μM ATP were immunoprecipitated and resolved by gel electrophoresis. The bands corresponding to pp60$^{v\text{-}src}$ were excised, reelectrophoresed in the presence of 5 (lanes 1, 3, and 5) or 50 (lanes 2, 4, and 6) ng of *S. aureus* V8 protease, and analyzed by autoradiography. The positions of unproteolyzed pp60$^{v\text{-}src}$ and the V8 proteolytic products corresponding to the amino-terminal 34,000-dalton (N34) and carboxy-terminal 26,000-dalton (C26) fragments are denoted in the margin.

determined were not significantly different from those obtained by using nondetergent-treated membranes.

Based on the data presented in Fig. 5, one might argue that amino-terminal tyrosine phosphorylation should be detected in intact cells, where steady-state levels of ATP are in the range of 1 to 2 mM. We therefore questioned whether the amino-terminal phosphorylation site(s) might be regulated by additional mechanisms, such as by dephosphorylation. Accordingly, membranes were phosphorylated with [γ-^{32}P]ATP for 15 min, EDTA was added to quench the kinase reaction, and the phosphorylated membranes were then incubated further. After various periods of time, pp60$^{v\text{-}src}$ was immunoprecipitated, and the amount of radiolabel remaining in each half of the molecule was quantitated by one-dimensional peptide mapping. Although radiolabel was lost from both regions as a function of time, the dephosphorylation of the amino-terminal domain of pp60$^{v\text{-}src}$ proceeded more rapidly than that of the carboxy terminus (Fig. 6). This implies that the amino-terminal site(s) is more susceptible to the action of phosphatases and suggests that phosphatases may function to limit the extent of amino-terminal pp60$^{v\text{-}src}$ phosphorylation both in membrane fractions and intact cells.

DISCUSSION

In this paper, we have demonstrated that pp60$^{v\text{-}src}$ can be phosphorylated while bound to plasma membrane vesicles. The extent of phosphorylation was greatly enhanced in the presence of vanadate. Incubation of membranes with micromolar concentrations of ATP resulted in phosphorylation of pp60$^{v\text{-}src}$ in the carboxy-terminal 26,000-dalton domain. With increasing ATP concentrations, additional sites of tyrosine phosphorylation appeared in the amino-terminal region. The amino-terminal phosphorylation reaction exhibited a higher K_m for ATP and a lower V_{max} and was more rapidly dephosphorylated when compared with phosphorylation at the carboxy terminus.

The use of membrane vesicles has provided a system in which the kinase activity of pp60$^{v\text{-}src}$ can be evaluated while the polypeptide is still anchored to the lipid bilayer. Several studies have shown that the proportion of labeled phosphotyrosine increases when [γ-^{32}P]ATP is added to membranes from RSV-infected cells compared with normal cells (16, 17, 34). Garber et al. (17) showed that pp60$^{v\text{-}src}$ could be phosphorylated in vitro in RSV-transformed chicken embryo fibroblast membranes. We have extented their initial finding by characterizing the kinetic parameters of pp60$^{v\text{-}src}$ phosphorylation in RSV-transformed vole cell membranes. Our results indicate that phosphatase and ATP hydrolytic activities must be considered for quantitative analysis of membrane kinase reactions. Of the numerous compounds assayed, only vanadate ions (Na_3VO_4) were highly effective in enhancing pp60$^{v\text{-}src}$ phosphorylation levels. This property is probably due to the ability of vanadate to inhibit ATPases (3, 35) and phosphotyrosyl-protein phosphatases (38). However, even in the presence of vanadate, it was difficult to quantitate steady-state levels of phosphate incorporation into pp60$^{v\text{-}src}$ due to residual activity of ATP hydrolyses. At low ATP concentrations, one could not determine whether the leveling off of the kinase reaction with time was due to saturation of available sites or was simply a reflection of the decreasing concentration of [γ-^{32}P]ATP. This situation precluded a precise determination of the stoichiometry of pp60$^{v\text{-}src}$ phosphorylation.

The region of pp60$^{v\text{-}src}$ responsible for anchoring the molecule to the plasma membrane has been mapped to the

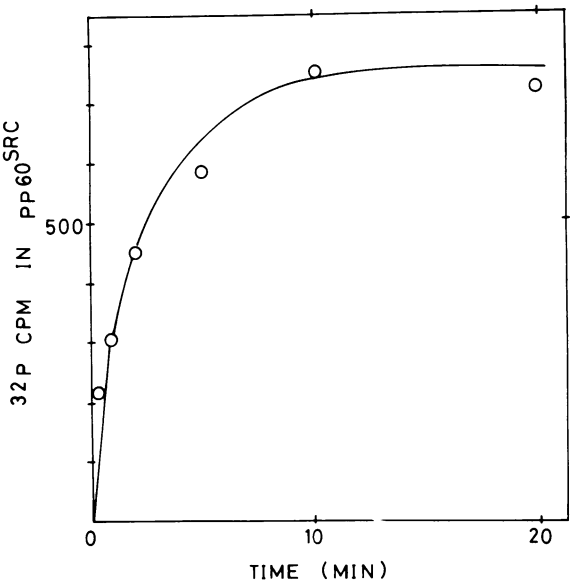

FIG. 4. Time dependence of phosphorylation of membrane-bound pp60$^{v\text{-}src}$. Membrane suspensions in 1.0 ml were incubated with 50 μCi of [γ-^{32}P]ATP (100 μM) and 1 mM vanadate at 22°C. At various times, the reaction was quenched and the amount of ^{32}P incorporated into pp60$^{v\text{-}src}$ was determined as described in the text.

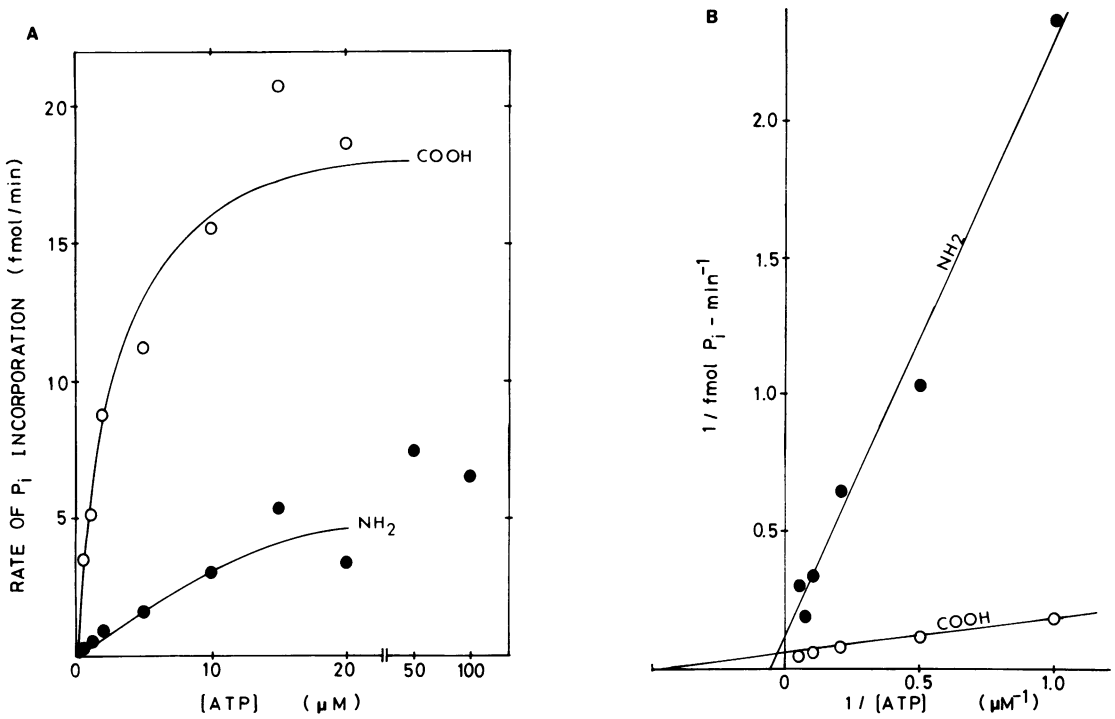

FIG. 5. Dependence of the rate of amino- and carboxy-terminal phosphorylation on the concentration of ATP. Membrane suspensions in 1.0 ml were incubated with concentrations of ATP ranging from 0.5 to 200 μM in the presence of 50 μCi of [γ-^{32}P]ATP and 1 mM vanadate for 1 min at 22°C (linear portion of the reaction, see Fig. 4). The amount of ATP remaining after 1 min was 85% of the total at 0.5 μM and 94% at 200 μM ATP. The reactions were quenched, immunoprecipitated, and gel electrophoresed. The pp60$^{v\text{-}src}$ band was reelectrophoresed in the presence of 20 ng of V8 protease, and the amounts of ^{32}P incorporated into the amino-terminal 34,000-dalton (●) and carboxy-terminal 26,000-dalton (○) fragments were quantitated. (A) The rate of phosphorylation as a function of ATP concentration is illustrated. The lines drawn through the data points represent the theoretical best fit based on linear regression analysis of the data in panel B. (B) The data in panel A are graphed in double-reciprocal form (Lineweaver-Burk plot). The data for amino (NH_2)- and carboxy (COOH)-terminal phosphorylation were analyzed by linear regression by the method of least squares (0.5 to 100 μM ATP). In this experiment, for the carboxy terminus, the K_m was 2.5 μM ATP, the V_{max} was 19 fmol/min, and the coefficient of determination, r^2, was 0.97. For the amino terminus, the K_m was 19 μM ATP, the V_{max} was 8 fmol/min, and r^2 was 0.98.

extreme amino terminus (12, 24). We were therefore interested in determining whether interaction of amino-terminal residues with the membrane prevented phosphorylation of tyrosine residues near this region. The data illustrated in Fig. 3 indicate that this is not the case. We suggest that the in vitro phosphorylation patterns of pp60$^{v\text{-}src}$ that we observed are mediated by the tyrosine kinase activity of pp60$^{v\text{-}src}$ itself, since antibody to the kinase domain effectively inhibits the phosphorylation reaction (Table 1). The appearance of amino-terminal phosphorylation sites in membrane-bound pp60$^{v\text{-}src}$ implies that this reaction may occur in vivo. The recent results of others (1, 5) with vanadate- or Mg^{2+}-treated cells support this conclusion.

It is tempting to compare the kinetic data reported here with that obtained with purified pp60$^{v\text{-}src}$ preparations. The K_m for autophosphorylation of purified pp60$^{v\text{-}src}$ is on the order of 25 μM (20). However, since phosphorylation of the amino and carboxy termini was not evaluated separately, it is difficult to make direct comparisons with the work cited here. It is interesting, however, that when purified pp60$^{v\text{-}src}$ is phosphorylated at high ATP concentrations, three to four times more phosphate is incorporated into the amino terminus than into the carboxy terminus (20). In membrane vesicles, this ratio was closer to one. This implies that in membranes only one phosphate molecule is incorporated into the amino-terminal region of the pp60$^{v\text{-}src}$ polypeptide and is consistent with the notion that interaction with the membrane partially restricts the extent of amino-terminal phosphorylation.

Finally, we have presented the first direct evidence that the amino-terminal phosphotyrosine residues are more susceptible to the actions of phosphoprotein phosphatases than those in the carboxy terminus. The fact that dephosphorylation of pp60$^{v\text{-}src}$ is observed even in the continued presence of vanadate (Fig. 6) is puzzling at first. However, it is possible that the presence of high concentrations of divalent cations (5 mM Mg^{2+}), in addition to functioning in the phosphorylation reaction, may also serve to inhibit phosphatase activity (5, 16). The addition of EDTA (time zero, Fig. 6) might thereby release this inhibition by chelation of the Mg^{2+} ions. Clearly, further characterization of the phosphatase species involved in this reaction is required.

The rapid dephosphorylation of amino-terminal phosphotyrosine residues observed in membrane-bound pp60$^{v\text{-}src}$ may explain why these sites are not observed when lysates are prepared from untreated, in vivo-labeled cells (6). Both our studies and those of Collett et al. (5) lend support to the hypothesis that the extent of pp60$^{v\text{-}src}$ phosphorylation in vivo may be regulated by the action of phosphatases. Genetic engineering experiments have established that phosphorylation of Ser-17 and Tyr-416 is dispensable for transformation (13, 37), but nothing is known about the role of the amino-terminal phosphotyrosine sites. It has been suggested that occupancy of these sites may serve to in-

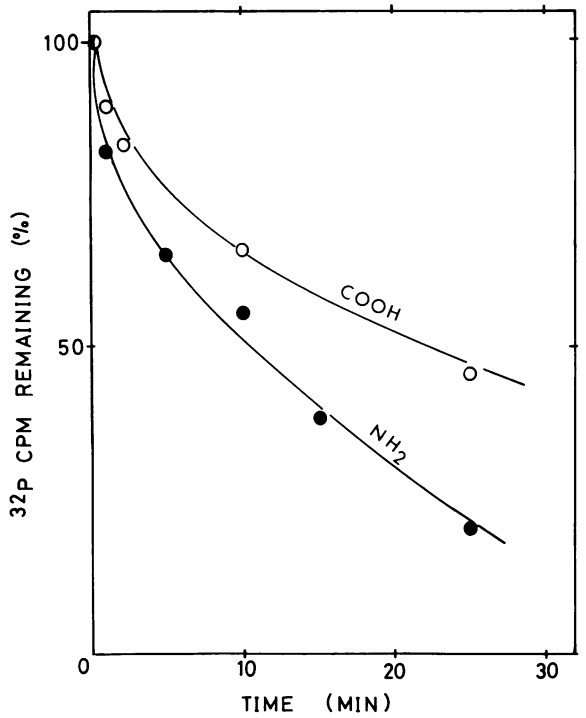

FIG. 6. Dephosphorylation of amino- and carboxy-terminal regions of pp60$^{v\text{-}src}$. The membrane suspension was incubated with [γ-^{32}P]ATP, 100 μM carrier ATP, and 50 μM vanadate for 15 min at 22°C. After the addition of 10 mM EDTA at time zero, portions were removed at various times (1 to 25 min), immunoprecipitated, and analyzed by one-dimensional peptide mapping as described in the legend to Fig. 5. The amounts of radioactivity incorporated into the amino (NH_2)-terminal (●) and carboxy(COOH)-terminal (○) fragments were approximately equal at this concentration of ATP (amino-terminal fragment: 990 cpm, carboxy-terminal fragment: 820 cpm), and were each normalized to 100% at time zero.

crease pp60$^{v\text{-}src}$ kinase activity (1, 5). Although the phosphorylation parameters of pp60$^{v\text{-}src}$ in our system are complex, we contend that membrane preparations are ideal systems in which to study the regulatory roles of phosphorylation and dephosphorylation on pp60$^{v\text{-}src}$ function.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant CA34943 from the National Institutes of Health and by a grant from the American Business Cancer Research Foundation. M.D.R. is a National Cancer Institute Postdoctoral Fellow (NRSA 5F32 CA07320) and R.L.E. is an American Cancer Society Professor of Cellular and Developmental Biology.

LITERATURE CITED

1. **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.
2. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
3. **Cantley, L. C., L. Josephson, R. Warner, M. Yanagisawa, C. Lechene, and G. Guidotti.** 1977. Vanadate is a potent (Na^+, K^+) ATPase inhibitor found in ATP derived from muscle. J. Biol. Chem. **252**:7421–7423.
4. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
5. **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.
6. **Collett, M. S., E. Erikson, and R. L. Erikson.** 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. J. Virol. **29**:770–781.
7. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus src gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021–2024.
8. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein, pp60^{src}, shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
9. **Collett, M. S., S. K. Wells, and A. F. Purchio.** 1983. Physical modification of purified Rous sarcoma virus pp60$^{v\text{-}src}$ protein after incubation with ATP/Mg^{2+}. Virology **128**:285–297.
10. **Courtneidge, S. A., and J. M. Bishop.** 1982. Transit of pp60^{src} to the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **79**: 7117–7121.
11. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus and a homologous protein in normal cells are associated with the plasma membrane. Proc. Natl. Acad. Sci. U.S.A. **77**:3783–3787.
12. **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.
13. **Cross, F. R., and H. Hanafusa.** 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine and serine phosphorylation of p60^{src} are dispensible for transformation. Cell **34**:597–607.
14. **Erikson, E., M. S. Collett, and R. L. Erikson.** 1978. In vitro synthesis of a functional avian sarcoma virus transforming gene product. Nature (London) **274**:919–921.
15. **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.
16. **Gallis, B., P. Bornstein, and D. L. Brautigan.** 1981. Tyrosyl protein kinase and phosphatase activities in membrane vesicles from normal and Rous sarcoma virus-transformed rat cells. Proc. Natl. Acad. Sci. U.S.A. **78**:6689–6693.
17. **Garber, E. A., J. G. Krueger, H. Hanafusa, and A. R. Goldberg.** 1983. Temperature-sensitive membrane association of pp60^{src} in tsNY68-infected cells correlates with increased tyrosine phosphorylation of membrane-associated proteins. Virology **126**:73–86.
18. **Gilmer, T. M., and R. L. Erikson.** 1981. Rous sarcoma virus transforming protein, p60^{src}, expressed in E. coli, functions as a protein kinase. Nature (London) **294**:771–773.
19. **Gilmer, T. M., and R. L. Erikson.** 1983. Development of anti-pp60^{src} serum with antigen produced in *Escherichia coli*. J. Virol. **45**:462–465.
20. **Graziani, Y., E. Erikson, and R. L. Erikson.** 1983. Characterization of the Rous sarcoma virus transforming gene product. J. Biol. Chem. **258**:6344–6351.
21. **Hanafusa, H.** 1977. Cell transformation by RNA tumor viruses, p. 401–483. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 10: viral gene expression and integration. Plenum Publishing Corp., New York.
22. **Hunter, T., and B. M. Sefton.** 1980. The transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
23. **Krueger, J. G., E. A. Garber, and A. R. Goldberg.** 1983. Subcellular localization of pp60^{src} in Rous sarcoma virus-transformed cells. Curr. Top. Microbiol. Immunol. **107**:52–124.
24. **Krueger, J. G., E. A. Garber, A. R. Goldberg, and H. Hanafusa.** 1982. Changes in amino-terminal sequences of pp60^{src} lead to decreased membrane association and decreased in vivo tumorigenicity. Cell **28**:889–896.
25. **Krueger, J. G., E. Wang, and A. R. Goldberg.** 1980. Evidence that the src gene product of Rous sarcoma virus is membrane

associated. Virology **101**:25–40.
26. **Krzyzek, R. A., R. L. Mitchell, A. F. Lau, and A. J. Faras.** 1980. Association of pp60^{src} and *src* protein kinase activity with the plasma membrane of nonpermissive and permissive avian sarcoma virus-infected cells. J. Virol. **36**:805–815.
27. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
28. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561–572.
29. **Levinson, A. D., H. E. Opperman, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. **255**:11973–11980.
30. **Purchio, A. F.** 1982. Evidence that pp60^{src}, the product of the Rous sarcoma virus *src* gene, undergoes autophosphorylation. J. Virol. **41**:1–7.
31. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus src gene. Proc. Natl. Acad. Sci. U.S.A. **75**: 1567–1571.
32. **Purchio, A. F., E. Erikson, and R. L. Erikson.** 1977. Translation of 35S and of subgenomic regions of avian sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. **74**:4661–4665.
32a. **Resh, M. D., and R. L. Erikson.** 1985. Highly specific antibody to Rous sarcoma virus src gene product recognizes a novel population of pp60$^{v\text{-}src}$ and pp60$^{c\text{-}src}$ molecules. J. Cell Biol. **100**:409–417.
33. **Rohrschneider, L. R.** 1980. Adhesion plaques of Rous sarcoma virus-transformed cells contain the src gene product. Proc. Natl. Acad. Sci. U.S.A. **77**:3514–3518.
34. **Shriver, K., and L. R. Rohrschneider.** 1981. Spatial and enzymatic interaction of pp60^{src} with cytoskeletal proteins in isolated adhesion plaques and junctions from RSV-transformed NRK cells. Cold Spring Harbor Conf. Cell Proliferation **8**:1247–1262.
35. **Simons, T. J. B.** 1979. Vanadate-a new tool for biologists. Nature (London) **281**:337–338.
36. **Smart, J. E., H. Opperman, A. P. Czernilofsky, A. F. Purchio, R. L. Erikson, and J. M. Bishop.** 1981. Characterization of sites for tyrosine phosphorylation in the transforming protein of Rous sarcoma virus (pp60$^{v\text{-}src}$) and its normal cellular homologue (pp60$^{c\text{-}src}$). Proc. Natl. Acad. Sci. U.S.A. **78**:6013–6017.
37. **Snyder, M. A., J. M. Bishop, W. W. Colby, and A. D. Levinson.** 1983. Phosphorylation of tyrosine-416 is not required for the transforming properties and kinase activity of pp60$^{v\text{-}src}$. Cell **32**:891–901.
38. **Swarup, G., S. Cohen, and D. L. Garbers.** 1982. Inhibition of phosphotyrosyl-protein phosphatase activity by vanadate. Biochem. Biophys. Res. Commun. **107**:1104–1109.
39. **Takeya, T., R. A. Feldman, and H. Hanafusa.** 1982. DNA sequence of the viral and cellular *src* gene of chickens. J. Virol. **44**:1–11.
40. **Wells, S. K., and M. S. Collett.** 1983. Specific proteolytic fragmentation of p60$^{v\text{-}src}$ in transformed cell lysates. J. Virol. **47**:253–258.
41. **Willingham, M. C., G. Jay, and I. Pastan.** 1979. Localization of the avian sarcoma virus src gene product to the plasma membrane of transformed cells by electron microscopic immunocytochemistry. Cell **18**:125–134.