Isolation of a catalytically competent phosphorylated tyrosine kinase from Rous sarcoma virus-induced rat tumor by immunoadsorption to and hapten elution from phosphotyrosine binding antibodies

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A procedure has been developed for the isolation of a catalytically competent phosphorylated tyrosine kinase (RSV Y-kinase) from avian sarcoma virus-induced rat tumors. The procedure involves reaction of partially purified RSV Y-kinase with ATP to effect tyrosyl phosphorylation of catalytically competent RSV Y-kinase. Tyrosyl phosphorylated RSV Y-kinase was isolated from the heterogeneous reaction mixture by immunoadsorption on immobilized phosphotyrosyl binding antibodies and elution with the hapten p-nitrophenyl phosphate. Estimation of the phosphate content of the purified phosphorylated RSV Y-kinase indicated that 1–3 tyrosyl groups had been phosphorylated upon reaction with ATP. The specific activity toward histone 2B of the purified phosphorylated RSV Y-kinase was at least 30-fold greater than that estimated for the RSV Y-kinase prepared previously by immunoadsorption on immobilized antiserum from tumor bearing rabbits.

Introduction

pp60vsrc, the Rous sarcoma virus (RSV)-transforming factor [1–4] is a kinase [3,5] that catalyzes phosphorylation of tyrosine residues in peptides and proteins (e.g., Ref. 6–9). This 60 kDa enzyme can also undergo autophosphorylation, primarily at Tyr-416 [10–11]. The observation that temperature-sensitive transformation-defective mutants of RSV exhibit reduced kinase activity and are not tyrosyl phosphorylated at the restrictive temperature, suggests that a kinase activity of pp60vsrc may be required for expression of oncogenic activity [5,9,12–13]. Moreover, Balb/c mouse 3T3 cells bearing RSV suffer an approx. 1000-fold decrease in tumorigenicity when wild-type RSV is replaced by a variant wherein Tyr-416 in pp60vsrc is replaced by Phe [14]. Additionally, deletion of Tyr-416 in pp60vsrc has been observed to decrease the rate of virus-mediated tumor induction in chicks [15]. Interestingly, wild-type pp60vsrc, the Phe-416 variant and variants of pp60vsrc, wherein Tyr-416 is deleted, have been reported to exhibit similar tyrosyl kinase activities toward anti-pp60vsrc immunoglobulins in assays where the src kinases were precipitated from crude extracts by the immunoglobulin substrates [15,16]. These observations suggest that either, (i) phosphorylation of Tyr-416 specifically alters the kinase activity of pp60vsrc toward a physiologically important substrate and thereby enhances its potency as a transforming factor; or (ii) phosphorylation of Tyr-416 alters interactions between pp60vsrc and proteins that exhibit an increased or diminished activity upon binding to pp60vsrc. Identification of the molecular basis for the ability of pp60vsrc to effect transformation will require characterization of the interactions of pure phosphorylated pp60vsrc with cellular proteins. In the present paper we describe a procedure for purification of phosphorylated RSV Y-kinase that involves adsorption of the tyrosine phosphorylated kinase on immobilized phosphotyrosine binding antibody and elution of catalytically competent protein with a hapten. The resulting material which should be...
suitable for biophysical studies has a specific enzymatic activity at least 30-fold greater than that estimated for the pp60^src prepared previously by immunoadsorption on immobilized antiserum from tumor-bearing rabbits.

Materials and Methods

Electrophoresis reagents were purchased from IBI and [γ-^{32}P]ATP (5000 Ci/mmol) from Amersham. HA-Ultragel was from LKB, DEAE-Sephaloc from Pharmacia and oligo(dA)cellulose from P.L. Biochemicals. D-Serine dehydratase and lipoyl dehydrogenase were the generous gifts of Dr. Michelle Marceau and Dr. Vincent Massey (University of Michigan). Antibody to pp60^src, prepared according to the method of Resh and Erikson [17], was kindly supplied by Dr. Raymond L. Erikson (Harvard University). Unless specified, other reagents were from Sigma or Fisher.

B31 cells, a clonal cell line of B77-avian sarcoma virus transformed rat-1 cells (F2408 line of Fischer rat embryo cells), were obtained from Dr. H.E. Varmus, University of California, San Francisco. Tumors were raised in 150-200 g male Fischer 344 rats by subcutaneous injection of 5.10^5 B31 cells into each flank. After 2-3 weeks, tumors were excised, washed in phosphate-buffered saline, frozen in liquid nitrogen and stored at -70°C until further use.

Partial purification of RSV Y-kinase

A modification and extension of the procedure of Blithe et al. [18] was used to obtain a partially purified enzyme fraction. Frozen tumor (40 g) was powdered under liquid nitrogen and homogenized directly in 100 ml of extraction buffer (40% glycerol, 4 mM EDTA, 0.2% Nonidet P-40 (NP-40), 100 mM KCl, 30 mM BME and 0.05% NP-40, 30 mM BME and 1 mM EDTA) and the retentate centrifuged for 10 min at 10,000 rpm in a Sorvall centrifuge using an SS-34 rotor. The supernatant was batch-loaded onto 125 ml of DEAE-Sephacl resin (equilibrated with buffer A) by slowly rotating the mixture end-over-end for 1 h. The resin was collected by centrifugation for 5 min at 1000 rpm using an IEC PR-6000 centrifuge. After two, 1 h washes (260 ml of buffer A used for each wash) kinase activity was eluted from the resin using 250 ml of buffer A containing 150 mM NaCl; both the washings and the elution were performed in the manner described for batch loading. After dilution with an equal volume of buffer A, the eluate was loaded at a rate of 40 ml/h onto a 14 ml (0.90 cm diameter x 22 cm) bed of oligo(dA)cellulose resin equilibrated with buffer A. The resin was washed with 3 column vol. of buffer A and protein eluted with a 50 ml linear gradient of 0-1 M KCl in buffer A containing 20 mM KPO_4 (pH 7). Pooled active fractions (27.5 ml) were dialyzed for 16 h against 30 vol. of protein A containing 1 mM EDTA and 100 mM KCl. The retentate was loaded onto a 79 ml (2.5 cm diameter x 16 cm) bed of HA-Ultragel resin equilibrated with buffer B (buffer A containing 1 mM EDTA). After washing the resin with 3 column vol. of buffer B, protein was eluted using a 200 ml linear gradient of 5-250 mM KPO_4 (pH 7) in buffer B. Pooled active fractions (26.5 ml) were dialyzed for 16 h against 30 vol. of enzyme storage buffer (5 mM KPO_4, 30% glycerol, 0.05% NP-40, 30 mM BME and 1 mM EDTA) and the retentate concentrated 10-fold in an Amicon ultrafiltration apparatus using a YM10 membrane. The resulting partially purified RSV Y-kinase was stored in liquid nitrogen.

Affinity purification of phosphorylated RSV Y-kinase

An affinity matrix for further purification of RSV Y-kinase was prepared by covalently crosslinking O-phosphotyrosine binding antibodies (anti-PY) [19] to 100 µl of protein A-Sepharose (3.8 µg antibody per µl protein A-Sepharose) by the method of Schneider et al. [20]. The immobilized anti-PY was then placed in a Beckman ultracentrifuge at 21,000 rpm for 180 min in an SW27 rotor.

Subsequent operations were conducted at 4°C. The supernatant was filtered through glass wool and applied at a rate of 40 ml/h to a 96 ml (2.5 cm diameter x 19.5 cm) bed of HA-Ultragel resin equilibrated with 5 mM KPO_4 (pH 7), 20% glycerol, 0.1% NP-40 and 30 mM BME. The resin was washed with 5 column vol. of equilibrating buffer and protein eluted with 250 ml of 250 mM KPO_4 (pH 7), 20% glycerol, 0.1% NP-40, 30 mM BME, 5 mM EDTA, 100 mM KCl and 100 Kallikrein units of aprotinin per ml. The eluate was dialyzed (using Spectra Por 2 dialysis tubing) for 18 h against 10 vol. of buffer A (5 mM KPO_4 (pH 7), 20% glycerol, 0.1% NP-40, 30 mM BME and 5 mM EDTA) and the retentate centrifuged for 10 min at 10,000 rpm in a Sorvall centrifuge using an SS-34 rotor. The supernatant was made up to 240 ml (0.90 cm diameter x 22 cm) bed of oligo(dA)cellulose resin equilibrated with buffer A. The resin was washed with 3 column vol. of buffer A and protein eluted with a 50 ml linear gradient of 0-1 M KCl in buffer A containing 20 mM KPO_4 (pH 7). Pooled active fractions (27.5 ml) were dialyzed for 16 h against 30 vol. of buffer A containing 1 mM EDTA and 100 mM KCl. The retentate was loaded onto a 79 ml (2.5 cm diameter x 16 cm) bed of HA-Ultragel resin equilibrated with buffer B (buffer A containing 1 mM EDTA). After washing the resin with 3 column vol. of buffer B, protein was eluted using a 200 ml linear gradient of 5-250 mM KPO_4 (pH 7) in buffer B. Pooled active fractions (26.5 ml) were dialyzed for 16 h against 30 vol. of enzyme storage buffer (5 mM KPO_4, 30% glycerol, 0.05% NP-40, 30 mM BME and 1 mM EDTA) and the retentate concentrated 10-fold in an Amicon ultrafiltration apparatus using a YM10 membrane. The resulting partially purified RSV Y-kinase was stored in liquid nitrogen.
aprotinin and 15 μl of immobilized anti-PY. The gel and liquid phase were mixed for 20 min at 4°C and then centrifuged to separate the gel and supernatant (S2) phases. (All mixing of gels and liquid phases was performed by end-over-end-rotation of vials containing the two phases.) The gel was washed with 140 μl of 0.05% NP-40, centrifuged, and the resulting supernatant combined with the supernatant from each sample was subjected to SDS-PAGE (11.3% gels).

**Immunoadsorption of RSV Y-kinase with antibodies to pp60<sup>5src</sup>**

An affinity matrix was prepared by mixing either 50 μg of pp60<sup>5src</sup> binding antibodies or 100 μg of normal rabbit IgG in a vial containing 15 μl of hydrated protein A-Sepharose and 20 μl buffer C for 1 h at 4°C. The gel was washed three times with buffer D (100 μl) and three times with buffer C (200 μl). A 10 μl sample of affinity-purified phosphorylated RSV Y-kinase was mixed at 4°C for 1 h (to effect immunoadsorption) with 15 μl of either immobilized antibody to RSV Y-kinase or immobilized rabbit IgG. The immobilized antibody was removed by centrifugation, and 10 μl of the supernatant from each sample was subjected to SDS-PAGE (8.5% gels).

**Kinase activity**

The kinase activity of RSV Y-kinase was determined with histone 2B as substrate. A 1 μl sample of the fraction to be assayed was incubated with 15 μl of 150 mM Mops (pH 6.7), 0.05% NP-40 and 2.5 mM BME for 15 min at room temperature. Phosphorylation was initiated with the addition of 9 μl of a solution containing histone 2B, [γ-<sup>32P</sup>]ATP and MgCl<sub>2</sub> to yield a final concentration of 20 μM histone 2B, 5 mM MgCl<sub>2</sub> and 50 μM [γ-<sup>32P</sup>]ATP (3000 dpm/pmol). After 20, 40 or 60 min (as indicated) 8 μl of quenching solution was added and the solution heated at 95°C for 5 min. The quenched samples (10 μl) were then subjected to SDS-PAGE (11.3% gels).

To allow comparison with published studies [22], the kinase activity was also determined by equilibrating 2.5 μl of affinity-purified phosphorylated RSV Y-kinase with 35 μl of 45 mM Mes (pH 6.5) and 0.07% NP-40 at 22°C for 15 min, whereupon 25 μl of a solution containing histone 2B, [γ-<sup>32P</sup>]ATP and MgCl<sub>2</sub> was added to yield a final concentration of 33 μM histone 2B, 5 mM MgCl<sub>2</sub> and 1 μM [γ-<sup>32P</sup>]ATP (3000 dpm/pmol). After 20, 40 and 60 min, 18 μl of the reaction mixture was added to 6 μl of quenching solution and heated for 5 min at 95°C. Following SDS-PAGE (10 μl samples) the comparative rate of phosphorylation was determined from the integrated absorbance of autoradiograms of the histone 2B bands.

**SDS-PAGE, protein content and phosphate incorporation**

SDS-PAGE was performed as described by Laemmli [23]. Solutions to be analyzed by SDS-PAGE were quenched with quenching solution and heated for 5 min at 95°C. Molecular mass standards used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (92 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa). Silver-stained gels were developed according to the method of Morrissey [24]. Autoradiograms were exposed at -70°C using XAR-5 film (Kodak). To estimate protein content and phosphate incorporation, a Zeineh soft laser scanning densitometer (Biomed Instrument, model SI-TRFF) was used to obtain absorbance measurements of the bands on the silver-stained gels and autoradiograms. Protein content was estimated from a linear standard curve relating integrated absorbance to protein content. The standard curve was prepared using 0.5–2 ng of N-seryl dehydroaspartate or lipoyl dehydrogenase as a reference standard. Phosphate incorporation into RSV Y-kinase and histone 2B was estimated from an autoradiogram. Standard curves for phosphate incorporation were prepared with the [γ-<sup>32P</sup>]ATP (3500 dpm/pmol) used to phosphorylate the partially purified RSV Y-kinase. Dilutions containing between 250 and 2000 dpm/μl were prepared and 1 μl of each dilution spotted on a blank region of the gel containing the RSV Y-kinase or histone 2B.
**Acid hydrolysis**

Phosphoserine and phosphothreonine were analyzed by a modification of the method of Hunter and Sefton [6]. Radioactive bands on dried Coomassie-stained gels were localized by autoradiography, excised, placed in a 1.5 ml polypropylene vial containing 40 μl of 100 mM NH₄HCO₃, ground to a fine powder (with a teflon pestle), suspended in 1.1 ml of 45 mM NH₄HCO₃ containing trypsin (0.9 mg/ml, Worthington) and incubated on a shaker at 37 °C. After 12 h the gel was separated from the aqueous phase by centrifugation and incubated with 1.1 ml of fresh trypsin/bicarbonate buffer for another 12 h. The aqueous phase from the second digest was separated from the gel, combined with that of the first digest and evaporated. The gel was washed with 100 μl of 45 mM NH₄HCO₃ containing 50 nmol of unlabelled phosphothreonine and phosphoserine. After centrifugation, the supernatant was combined with the residue from the aqueous phases and evaporated. The residue was quantitatively transferred to a Pyrex tube with 150 μl of water, the water evaporated and the residue resuspended in 200 μl of constant boiling 6 M HCl (Pierce). The tube was placed in a gas tight vial (Pierce) and purged with N₂ (1 atm). After hydrolysis at 110 °C for 5 h, the acid was evaporated and the residue taken up in 150 μl of water, transferred to a 1.5 ml polypropylene centrifuge tube and again taken to dryness under reduced pressure. The resulting residue was taken up in 0.2 ml of 0.1 M formic acid and processed on a 0.2 ml column of Dowex 50 (Bio Rad) according to the method of Martensen [25]. The fraction that eluted with formic acid, which contained phosphoserine and phosphothreonine but not phosphotyrosine (phosphotyrosine is not eluted from the column by the formic acid) was taken to dryness, the residue taken up in 5 μl water, spotted on cellulose TLC plates (Whatman) and electrophoresed at pH 3.5 (pyridine/acetic acid/water (1 : 10 : 189 v/v)) for 1 h at 50 V/cm, 0 °C. Phosphoamino acid reference standards were visualized with ninhydrin and radioactive spots were localized by autoradiography.

**Base hydrolysis**

Phosphotyrosine was analyzed by a modification of the method of Martensen and Levine [26]. Radioactive bands from dried Coomassie-stained gels were placed in teflon inserts in reactivials (Pierce) with BSA (0.5 mg/ml) and 5 M KOH (total volume 200 μl) and hydrolyzed at 155 °C for 70 min. After transfer to polypropylene vials the hydrolysate was neutralized with 10 M HClO₄ and the resulting KClO₄ precipitate was removed by centrifugation. The remaining supernatant was made 0.2 M in formic acid and treated with 0.2 ml Dowex [25]. The Dowex resin was washed with 0.1 M formic acid so that the total volume of formic acid solution that passed through the column was 1.1 ml. Phosphotyrosine was eluted from the Dowex 50 with 0.9 ml of water. The water was evaporated and the residue taken up in 5 μl of water and subjected to electrophoresis on cellulose plates as described for acid hydrolysis.

**Results and Discussion**

RSV Y-kinase was partially purified from tumors raised in Fisher 344 rats by chromatography on hydroxyapatite, DEAE-Sepharose and oligo(dA)cellulose using procedures similar to those described by Blithe et al. [18]. A solution of the partially purified RSV Y-kinase was treated with anti-PY to remove endogenous tyrosyl phosphorylated proteins and reacted with [γ-³²P]ATP to effect phosphorylation of RSV Y-kinase. Although the partially purified phosphorylated RSV Y-kinase was highly heterogeneous (Fig. 1A, lane A), highly purified tyrosyl phosphorylated RSV Y-kinase could be isolated from this mixture by adsorption to and elution from immobilized anti-PY. Fig. 1A (lane C) shows that the fraction eluted from the immobilized antibody by the hapten pNPP was highly purified and consisted almost entirely of protein which comigrated on SDS-PAGE with proteins in the molecular weight range 57000-58000. Comparison of lane C in panels A and B of Fig. 1 showed that the ³²P-labeled material in the eluent comigrated with the major silver-staining bands, consistent with the well documented autophosphorylation of pp60⁺⁻. The Mᵣ of 57000-58000 for the RSV Y-kinase is close to that expected for pp60⁺⁻ and to the value of 54000 observed by Blithe et al. [18] for the RSV Y-kinase purified from rat tumors by adsorption to and elution from immobilized IgG from TBR sera. The lower than expected Mᵣ observed by Blithe et al. [18] for RSV Y-kinase was attributed to proteolytic nicking near the N-terminus. It is not clear whether the higher molecular weight observed for our preparation of RSV Y-kinase reflects less extensive proteolysis of RSV Y-kinase or experimental error.

The identity of the 57 and 58 kDa bands as RSV Y-kinase was verified by the observation (Fig. 2) that antibody raised against pp60⁺⁻ (that had been expressed by Escherichia coli) immunoprecipitated ³²P-labeled material (from affinity purified phosphorylated RSV Y-kinase) that migrated with Mᵣ values of 57000 and 58000. The presence of two cross-reacting bands migrating with Mᵣ values of 57000 and 58000 (Fig. 1, panel A) is reminiscent of previous observations of Collett et al. [27]. These investigators noted that incubation of partially purified RSV Y-kinase with ATP at concentrations near or above the Kₘ (7-36 μM [8,20,28-29]) led to the production of two phosphorylated species in roughly equivalent amounts which migrated as two closely spaced bands on SDS-PAGE. The band with the lower electroforetic mobility was more heavily phosphorylated than the faster moving.
band which comigrated with unphosphorylated RSV Y-kinase. The structural basis for the decreased mobility of the more heavily phosphorylated species remains to be established as does the reason for the inability of Collett et al. [27] to obtain more than a 50% yield of the

more heavily phosphorylated derivative. Examination of the autoradiogram in Fig. 2 reveals that affinity purified phosphorylated RSV Y-kinase contains a small amount of phosphorylated protein migrating with a molecular mass of 66 kDa. A similar impurity, which like the 66 kDa protein did not precipitate with pp60 vinc binding serum, was reported by Neer and Lok [30] as a 64 kDa contaminant in their preparation of a pp60 vinc related tyrosine kinase from bovine brain. As pointed out by Neer and Lok [30] the contaminant may be a tyrosine kinase or a substrate for one.

It is important to note that purification occurred during both the adsorption and elution steps in the treatment of phosphorylated RSV Y-kinase with immobilized anti-PY. Phosphorylated RSV Y-kinase was specifically eluted from anti-PY by hapten (compare lanes B and C of Fig. 1). The yield of adsorbed RSV Y-kinase recovered by elution with the hapten was 47%. Although several proteins were adsorbed on the immobilized anti-PY, only phosphorylated RSV Y-kinase appeared to be eluted from the resin by the hapten (compare lanes C and G of Fig. 1). The observation that little additional phosphorylated RSV Y-kinase was obtained after a second immunoadsorption with additional immobilized anti-PY suggests that the first immunoadsorption step captured most of the phosphorylated RSV Y-kinase (compare Fig. 1, lanes C and F).

Control experiments (data not shown) showed that when the starting material for affinity chromatography was not treated with anti-PY prior to incubation with ATP the yield of the 57–58 kDa protein was not altered. This observation suggests that prior to addition of ATP little
of the RSV Y-kinase in the partially purified material contains phosphorylated tyrosine residues.

A standard curve relating the amount of silver-stained protein standards to integrated absorbance, and a standard curve that related the amount of a [γ-32P]ATP standard to the integrated absorbance of an autoradiogram, were used to estimate the extent of incorporation of phosphate into RSV Y-kinase from the integrated absorbance of RSV Y-kinase on silver-stained gels and autoradiograms. Assuming the response of RSV Y-kinase to the silver stain is the same as that of a lipoil dehydrogenase or D-serine dehydratase protein standard, the radioactivity of the RSV Y-kinase band as judged from the intensity of the autoradiogram indicated 1.4 (lipoil dehydrogenase standard) or 2.8 (D-serine dehydratase standard) phosphoryl groups were incorporated per molecule of RSV Y-kinase. Thus, the variation of proteins with respect to their response to silver stain introduces considerable uncertainty in estimates of the extent of phosphorylation of RSV Y-kinase. Phosphoamino acid analysis after limited acid and base hydrolysis of the affinity purified RSV Y-kinase indicated that it had been phosphorylated almost exclusively on tyrosine residues upon treatment with [γ-32P]ATP. (compare Fig. 4, panels A and B, lane A). Fig. 3 shows that most of the histone kinase activity in the starting material for affinity purification of phosphorylated RSV Y-kinase did not bind to the immobilized anti-PY (compare lanes A, C and D). The observation that the unadsorbed material phosphorylated histone primarily on serine and threonine, whereas the phosphorylated RSV Y-kinase phosphorylated histone almost exclusively on tyrosine (compare lanes B and C).
of panels A and B, Fig. 4) supports the conclusion (see above) that the immobilized anti-PY adsorbed essentially all of the RSV Y-kinase.

Fig. 5 depicts time dependencies for the incorporation of phosphate into histone 2B by affinity purified phosphorylated RSV Y-kinase in the presence of 50 

µM ATP (O) as described under Materials and Methods. At the indicated times, aliquots of the assay mixtures were quenched and 10

µl of each quenched sample was subjected to SDS-PAGE. An autoradiogram of the dried gel was analyzed using a soft laser densitometer.

Fig. 5. Activity of phosphorylated RSV Y-kinase toward histone 2B. Phosphorylations were catalyzed by 0.87 ng of affinity-purified phosphorylated RSV Y-kinase in the presence of either 50 µM ATP (O) or 1 µM ATP (x) as described under Materials and Methods. The assay was incubated for 10 min at 30 °C before the reaction was quenched with 10 µl of 5% trichloroacetic acid. The radioactivity was determined by liquid scintillation counting.

The observation that a 50-fold increase in the ATP concentration (from 1 to 50 µM) produced only a 2.8-fold increase in rate, suggests that at 50 µM ATP phosphorylated RSV Y-kinase is nearly saturated with respect to ATP. This conclusion is consistent with the values of 7–36 µM reported for the K_m for ATP for RSV Y-kinase [8,22,28,29]. If the phosphorylated RSV
Y-kinase were saturated with respect to ATP and histone in the presence of 50 μM ATP and 20 μM histone, the velocity observed under these conditions would indicate a turnover number of 0.051 s⁻¹ (1.1-2.8/60). If the $K_m$ for histone is well above 20 μM the ratio of the velocity at saturating ATP to the histone concentration would yield an estimate of $2.6 \times 10^3$ M⁻¹ s⁻¹ for the specificity constant ($k_{cat}/K_m$) for phosphorylated RSV Y-kinase complexed with ATP. In contrast to these values, the corresponding turnover number and specificity constant of hexokinase (with ATP and glucose as substrates) are 1300 s⁻¹ and $1.3 \times 10^7$ M⁻¹ s⁻¹, respectively [35]. The observation that the activity of phosphorylated RSV Y-kinase (toward histone 2B) is 3–4 orders of magnitude below that of hexokinase (toward glucose) may simply reflect the fact that histone 2B is not the physiological substrate of phosphorylated RSV Y-kinase. Hopefully, the method in this study for isolation of forms of RSV Y-kinase competent to undergo autophosphorylation will facilitate identification of physiologically important substrates of pp60^src.

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