

V_{\max} activation of pp60^{c-src} tyrosine kinase from neuroblastoma Neuro-2A

Wayne T. MATTEN and Patricia F. MANESS*

Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, NC 27514, U.S.A.

A kinetic analysis of the tyrosine-specific protein kinase of pp60^{c-src} from the C1300 mouse neuroblastoma cell line Neuro-2A and pp60^{c-src} expressed in fibroblasts was carried out to determine the nature of the increased specific activity of the neuroblastoma enzyme. In immune-complex kinase assays with ATP-Mn²⁺ and the tyrosine-containing peptide angiotensin I as phosphoacceptor substrate, pp60^{c-src} from the neuroblastoma cell line was characterized by a maximum velocity (V_{\max}) that was 7–15-fold greater than the V_{\max} of pp60^{c-src} from fibroblasts. The neuroblastoma enzyme exhibited K_m values for ATP ($16 \pm 3 \mu\text{M}$) and angiotensin I ($6.8 \pm 2.6 \text{ mM}$) that were similar to K_m values for ATP ($25 \pm 3 \mu\text{M}$) and angiotensin I ($6.5 \pm 1.7 \text{ mM}$) of pp60^{c-src} from fibroblasts. pp60^{v-src} expressed in Rous-sarcoma-virus-transformed cells exhibited an ATP K_m value ($25 \pm 4 \mu\text{M}$) and an angiotensin I K_m value ($6.6 \pm 0.5 \text{ mM}$) that approximated the values determined for pp60^{c-src} in neuroblastoma cells and fibroblasts. These results indicate that the pp60^{c-src} kinase from neuroblastoma cells has a higher turnover number than pp60^{c-src} kinase from fibroblasts, and that the neural form of the enzyme would be expected to exhibit increased catalytic activity at the saturating concentrations of ATP that are found intracellularly.

INTRODUCTION

Proto-oncogenes are intensively studied because of their presumed roles in normal cell growth and development, in addition to their potential role in helping understand the processes of cell transformation. The function of the cellular *src* (*c-src*) proto-oncogene is unknown, but several lines of evidence point to a role in neuronal cells. The protein product of *c-src* is a 63 kDa protein (pp60^{c-src}) with tyrosine-specific protein kinase activity [1–3]. pp60^{c-src} is preferentially synthesized in neural tissues, with highest concentrations expressed in embryonic neural retina and brain [4,5]. In neural retina and cerebellum pp60^{c-src} is expressed in neuronal cells at the onset of terminal differentiation when proliferation ceases, suggesting a role more closely linked to differentiation or function of neuronal cells than proliferation [6,7]. Immunocytochemical localization studies of pp60^{c-src} in sensory neurons of chick dorsal-root ganglion cultures [8] and biochemical identification of pp60^{c-src} in primary neuronal cultures from rat cerebral cortex [9] confirm that pp60^{c-src} is a product of neuronal cells. However, pp60^{c-src} is not restricted to neurons, as it is also expressed in neuroectodermal cells of early embryos [10], fibroblasts [1,11,12] and astroglial-cell cultures [9].

It has been established that the pp60^{c-src} protein kinase in neurons and neuroblastoma cells has an increased specific activity compared with pp60^{c-src} in fibroblasts and glial cells. Primary cultures of rat cortical neurons express a structurally modified form of pp60^{c-src} with specific kinase activity 6–12-fold greater than astrocytes from the same tissue, measured in the presence of subsaturating substrate concentrations [9]. Human neuroblastoma cell lines also produce a form of pp60^{c-src} with a 20–40-fold higher specific tyrosine kinase activity than that of pp60^{c-src} human glioblastoma cell lines or fibroblasts [13].

To investigate the basis for the higher pp60^{c-src} kinase specific activity in cells of neural origin, we carried out a kinetic analysis of the protein kinase activity of pp60^{c-src} from the C1300 mouse neuroblastoma cell line Neuro-2A and a mouse fibroblast line that overexpresses pp60^{c-src}. The Neuro-2A cell line is a clone derived from the spontaneously arising mouse neuroblastoma C1300 of sympathetic origin [14–16] that maintains differentiated neuronal functions, as indicated by neuronal morphology and expression of tyrosine hydroxylase [17]. The NIH(pMcsrc/cos)A cell line is a clone of NIH 3T3 mouse fibroblasts that overexpresses pp60^{c-src} from a cloned chicken *c-src* gene introduced into the cells by transfection [18]. We demonstrate in the present paper that pp60^{c-src} from the neuroblastoma cell line is a tyrosine-specific protein kinase, whose phosphotransferase activity is characterized by a V_{\max} that is 7–15-fold higher than that of pp60^{c-src} in the fibroblast cell line with only minor differences in K_m values for the phospho-donor or phospho-acceptor substrates. These results suggest that the neural form of pp60^{c-src} has a higher turnover number, allowing it to operate with enhanced catalytic efficiency even at the saturating concentrations of ATP present in cells.

EXPERIMENTAL

Cell culture and method of extraction

The mouse C1300 neuroblastoma Neuro-2A cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The NIH(pMcsrc/cos)A cell line was obtained from David Shalloway (Pennsylvania State University). The RR1022 cell line (American Type Culture Collection) is a rat tumour cell expressing the product of the *src* gene of Rous sarcoma virus (Schmidt–Ruppin strain). Neuro-2A neuroblastoma cells were

* To whom correspondence should be addressed.

grown in 100 mm-diam. tissue-culture plates to 80% confluence for no more than seven passages in Dulbecco's modified Eagle's medium containing 4.5 mg of glucose/ml, 15 mM-Hepes, 50 μ g of gentamicin/ml and 10% (v/v) fetal-calf serum (KC Biologicals, Lenexa, KS, U.S.A.) at 37 °C in a CO₂/air (1:19) atmosphere. NIH(pMsrc/cos)A and RR1022 cells were grown under the same conditions, substituting 5% calf serum (KC Biologicals) for fetal-calf serum.

For preparation of extracts, cells in tissue-culture dishes were washed three times in 0.15 M-NaCl/10 mM-potassium phosphate buffer, pH 7.0, and were lysed at 4 °C in 1 ml of lysis buffer, which contained 20 mM-potassium phosphate buffer, pH 7.0, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.15 M-KCl, 2 mM-sodium EDTA, 10 mM-NaF, 1 mM-sodium ATP, 200 μ M-sodium orthovanadate, 100 kallikrein-inhibitor units of aprotinin/ml and 2 mM EGTA or 0.01% (w/v) leupeptin (Sigma Chemical Co.). After 20 strokes in a Dounce homogenizer, lysates were centrifuged at 10000 *g* for 30 min and the supernatants retained. The resulting cell extracts were stored at -70 °C. No change in protein kinase activity resulted from storage at -70 °C. Protein concentrations were determined by the Lowry method [19].

Immune-complex protein kinase assays

pp60^{c-src} phosphotransferase activity was determined by the immune-complex protein kinase assay with human angiotensin I acetate salt (Sigma Chemical Co.) as phospho-acceptor substrate and [γ -³²P]ATP-Mn²⁺ as phospho-donor. pp60^{c-src} was immunoprecipitated from cell extracts under conditions of antibody excess with mouse monoclonal antibody 327 [20]. This antibody was raised against bacterially expressed pp60^{v-src} and was obtained from Joan Brugge (State University of New York at Stony Brook). Monoclonal antibody 327 recognizes specifically the avian and mammalian forms of pp60^{c-src} in normal uninfected cells, and pp60^{v-src} in RSV-transformed cells [20]. Cell extracts (1.4 mg in 1 ml of lysis buffer) were incubated for 2 h at 4 °C with 2 μ l of ascites fluid (0.1 mg/ml) containing monoclonal antibody 327. Then 50 μ l of 50% (v/v) goat anti-(mouse IgG) antibody-agarose (Sigma Chemical Co.) in lysis buffer was added to precipitate the immune complex. Immunoprecipitates were centrifuged in a microcentrifuge and washed four times in lysis buffer, once in 60 mM-Tris/HCl buffer, pH 7.2, containing 2.5 M-KCl, 2 mM-sodium EDTA, 10 mM-NaF, 50 μ M-sodium orthovanadate, 2 mM-EGTA and 100 kallikrein-inhibitor units of aprotinin/ml, and twice in 20 mM-Tris/HCl buffer, pH 7.2, containing 5 mM-MnCl₂, 50 μ M-sodium orthovanadate, 100 kallikrein-inhibitor units of aprotinin/ml and 0.1% (w/v) leupeptin. For measurement of specific activity, immunoprecipitates were divided into two equal portions. One half was used to measure kinase activity in the immune-complex protein kinase assay and the other half was used for immunoblotting to determine the amount of pp60^{c-src} protein. For measurement of kinase activity, the washed pellets were prewarmed to 30 °C and protein kinase reactions were initiated by adding a prewarmed solution (25 μ l) composed of 20 mM-Tris/HCl buffer, pH 7.2, containing 5 mM-MnCl₂, 5 mM-dithiothreitol, 50 μ M-sodium orthovanadate, 0.286 mCi of [γ -³²P]ATP (86 Ci/mmol; New England Nuclear)/ml and various concentrations of unlabelled sodium ATP

and angiotensin I, which were determined spectrophotometrically. Reactions were carried out for 20 min at 30 °C. Pilot experiments, which were carried out with pp60^{c-src} from each cell type to establish initial-velocity conditions, showed that angiotensin I phosphorylation was linear with respect to time and extract concentration for at least 30 min at 30 °C. Reactions were terminated by chilling the tubes briefly on ice, centrifuging in a microcentrifuge to remove immune complexes containing the enzyme, and transferring supernatants to new tubes. Phosphorylated angiotensin I in the supernatant was separated from reactants by binding to Whatman P81 phosphocellulose paper under acidic conditions as described by Glass *et al.* [21]. Briefly, 40 μ l of 5% (w/v) trichloroacetic acid was incubated with 20 μ l of the supernatant containing phosphorylated angiotensin I for 10 min on ice. Samples (40 μ l) of the acidified solutions were applied to 2 cm \times 2 cm squares of Whatman P81 paper, washed three times in 0.425% (v/v) H₃PO₄, dried, and counted for ³²P radioactivity in Omnifluor (Dupont) in a scintillation counter.

To obtain the initial-velocity values (*v*) used for determining kinetic constants, the total units of kinase activity in the immunoprecipitates (pmol/min) were divided by the relative amount of pp60^{c-src} in the immune complexes determined by immunoblotting. The amount of pp60^{c-src} in the immune complexes was quantified in absorbance units by densitometric scanning of the 60 kDa band on autoradiograms of immunoblots developed with monoclonal antibody 327. Immunoblotting of immunoprecipitates from neuroblastoma Neuro-2A and NIH(pMsrc/cos)A cell extracts was performed on the same nitrocellulose filter to obtain absorbance units reflecting the relative amounts of pp60^{c-src} protein.

Western immunoblotting

Washed immunoprecipitates were prepared as described for the immune-complex protein kinase assay, heated at 95 °C for 1 min in SDS sample buffer without a thiol reducing agent, then centrifuged in a microcentrifuge. Supernatants were electrophoresed on 7.5% polyacrylamide gels (9 cm) containing a 1/37 weight ratio of bisacrylamide to acrylamide with the Laemmli system [22]. Non-reducing conditions were used because IgG heavy chains (53 kDa) are recognized by the ¹²⁵I-labelled secondary antibody, interfering with the detection of pp60^{c-src} under reducing conditions. Proteins in the gels were electroblotted on to a nitrocellulose membrane (9 cm \times 12 cm) (Millipore) [23]. The nitrocellulose blot was incubated with a 1:20 dilution of monoclonal antibody 327 in a 0.8 ml volume of 50 mM-Tris/HCl buffer, pH 7.2, containing 150 mM-NaCl and 0.5% (v/v) Tween-20 (Sigma Chemical Co.) [24] for 1 h at room temperature. The blot was washed five times (200 ml) in the same buffer, incubated for 1 h at room temperature with 1 μ Ci of ¹²⁵I-labelled anti-[mouse IgG F(ab')₂ fragment] antibody (5–20 Ci/mmol; Amersham/Searle)/ml, washed as before and air-dried. ¹²⁵I-labelled pp60^{c-src} bands were detected by autoradiography on Dupont Cronex 6-plus X-ray film with Dupont Lightning Plus intensifying screens and quantified with an LKB 2202 Ultrascan laser densitometer. The time of film exposure was adjusted so that the intensity of an exposed region of film was within the linear range of response of the film to ¹²⁵I, as standardized previously [25].

Densitometric scanning of Western immunoblots

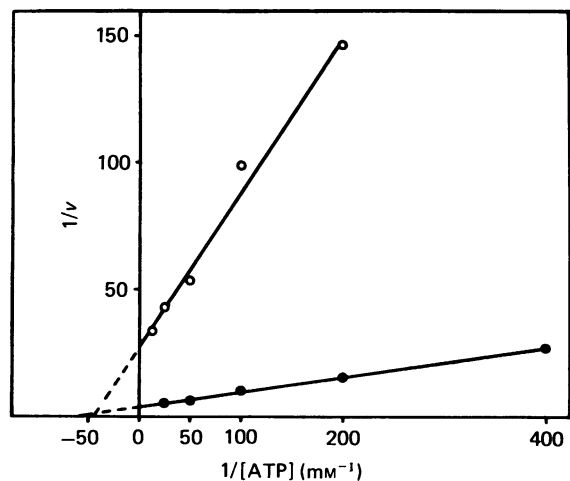


Fig. 1. Lineweaver-Burk plot of pp60^{c-src} tyrosine kinase activity from neuroblastoma Neuro-2A and fibroblasts in immune-complex protein kinase assays with various concentrations of ATP and a fixed concentration of angiotensin I (20 mM)

Initial velocity (v) indicates the number of pmol of phosphate transferred to angiotensin I/min by an equal amount of pp60^{c-src} protein in the immune complexes from extracts of both cell types. ●, pp60^{c-src} from neuroblastoma Neuro-2A cells; ○, pp60^{c-src} from NIH(pMcsrc/cos)A fibroblasts.

showed that there was approx. 20-fold more pp60^{c-src} in immunoprecipitates from 1.4 mg of NIH(pMcsrc/cos)A-cell extract (14600 absorbance units/mg of extract protein) compared with the neuroblastoma extract (714 absorbance units/mg of extract protein). It was necessary to employ a 20-fold difference in amount of pp60^{c-src} from the two cell types in the immune-complex protein kinase assays to obtain approximately the same number of units of protein kinase activity. This ensured reliability in the measurements of total ³²P radioactivity (c.p.m.) transferred to angiotensin I at low ATP concentrations.

No difference in specific activity of pp60^{c-src} immunoprecipitated from NIH(pMcsrc/cos)A-cell extracts was observed when extract concentrations were varied from 0.1 mg to 1.4 mg per ml of lysis buffer or when Neuro-2A extract concentrations were varied from 0.7 mg to 1.4 mg per ml in immune-complex kinase assays carried out at 20 μM-ATP-Mn²⁺ and 10 mM-angiotensin I. The amount of pp60^{c-src} determined by

immunoblotting was linear within this range of protein concentrations.

Determination of enzyme kinetic constants

K_m and V_{max} values and their standard errors were computed by the weighted least-squares method of Wilkinson [26]. The initial velocities and V_{max} values are relative values expressed as pmol of phosphate transferred to angiotensin I/min by 1 absorbance unit of pp60^{c-src}. For determining background values, lysis buffer was substituted for cell extract, and immune-complex protein kinase assays were carried out at the same fixed-substrate concentration and highest varied-concentration-substrate concentration used in the assay. Results of duplicate determinations were averaged and background values subtracted. At 40 μM-ATP the amount of ³²P transferred to angiotensin I in 20 min by pp60^{c-src} from the neuroblastoma Neuro-2A-cell extract (1.4 mg) was 50710 c.p.m., of which 5650 c.p.m. represented the background. At 40 μM-ATP the amount of ³²P transferred to angiotensin I in 20 min by pp60^{c-src} from the NIH(pMcsrc/cos)A-cell extract (1.4 mg) was 61620 c.p.m., of which 5080 c.p.m. represented the background.

RESULTS

To determine if pp60^{c-src} in mouse neuroblastoma Neuro-2A cells exhibited elevated tyrosine protein kinase activity, we determined K_m and V_{max} values for ATP and angiotensin I in immune-complex protein kinase reactions catalysed by pp60^{c-src} from neuroblastoma Neuro-2A cells and from NIH(pMcsrc/cos)A fibroblasts. These assays used monoclonal antibody 327 raised against bacterially expressed pp60^{v-src} that recognizes the mammalian and avian forms of pp60^{c-src} in normal untransformed cells [20]. pp60^{c-src} was immunoprecipitated from Neuro-2A-cell and NIH(pMcsrc/cos)A-cell extracts containing the same amount and concentration of protein. Phosphotransferase activity was measured with ATP-Mn²⁺ as phospho-donor and synthetic human angiotensin I peptide as phospho-acceptor substrate. Western immunoblots of immunoprecipitates from both cell types revealed a single major band at about 60 kDa, the expected size of pp60^{c-src}.

In the first experiment K_m and V_{max} values were determined with various concentrations of ATP-Mn²⁺ and a fixed concentration of human angiotensin I peptide (20 mM) that was near saturating. Mn²⁺ was used as bivalent cation because the activity of pp60^{c-src} kinase

Table 1. Kinetic constants of pp60^{c-src} kinase from neuroblastoma cells and fibroblasts

Values for ATP were obtained at 20 mM-angiotensin I, and those for angiotensin I at 100 μM-ATP-Mn²⁺. V_{max} is expressed as pmol of phosphate transferred to angiotensin I/min by an equal amount of pp60^{c-src} protein immunoprecipitated from Neuro-2A-cell or NIH(pMcsrc/cos)A-cell extract as determined by Western immunoblotting. Values are given as means ± S.E.M.

Cell line	ATP		Angiotensin I	
	K_m (μM)	V_{max}	K_m (mM)	V_{max}
Neuroblastoma (Neuro-2A)	16 ± 3	273 ± 23	6.8 ± 2.6	357 ± 59
Fibroblast [NIH(pMcsrc/cos)A]	25 ± 3	39 ± 2	6.5 ± 1.7	23 ± 2

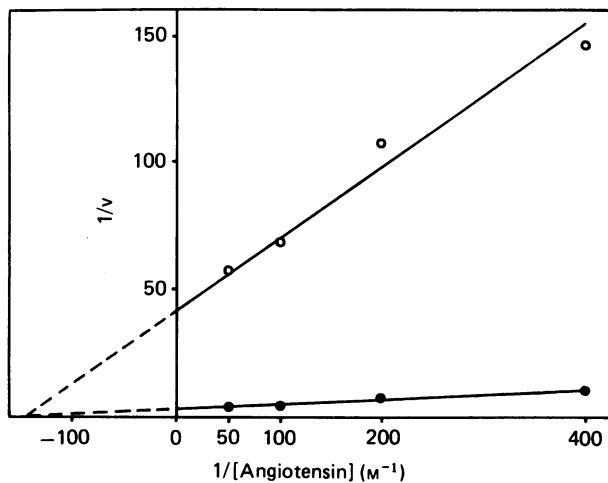


Fig. 2. Lineweaver-Burk plot of pp60^{c-src} tyrosine kinase activity from neuroblastoma Neuro-2A and fibroblasts in immune-complex protein kinase assays with various concentrations of angiotensin I and a fixed concentration of ATP (100 μ M)

Initial velocity (v) indicates the number of pmol of phosphate transferred to angiotensin I/min by an equal amount of pp60^{c-src} protein in the immune complexes from extracts of both cell types. ●, pp60^{c-src} from neuroblastoma Neuro-2A cells; ○, pp60^{c-src} from NIH(pMcsrc/cos)A fibroblasts.

was found to be significantly greater with Mn²⁺ than Mg²⁺, in agreement with other reports [27]. Michaelis-Menten plots of initial velocity (v) versus ATP concentration ($[S]$) showed that pp60^{c-src} from neuroblastoma Neuro-2A cells and fibroblasts exhibited typical saturation kinetics, with no observable allosteric effect on kinase activity by ATP within the range of ATP concentrations used. Lineweaver-Burk plots revealed that pp60^{c-src} kinase from Neuro-2A neuroblastoma cells had a maximum velocity that was 7-fold greater than that of pp60^{c-src} from fibroblasts (Fig. 1 and Table 1). In contrast, only very slight differences in ATP K_m values were observed between pp60^{c-src} from neuroblastoma cells (16 μ M) and fibroblasts (25 μ M) (Fig. 1 and Table 1).

In the second experiment K_m and V_{max} values were determined with various concentrations of angiotensin I and a near-saturating concentration of ATP-Mn²⁺ (100 μ M). Michaelis-Menten plots of initial velocity (v) versus angiotensin I concentration ($[S]$) showed that the forms of pp60^{c-src} kinase from neuroblastoma Neuro-2A cells and fibroblasts exhibited saturation kinetics with no observable allosteric effect on kinase activity by angiotensin I. Lineweaver-Burk plots revealed no significant difference in the angiotensin I K_m values for pp60^{c-src} in the two cell types (Fig. 2 and Table 1). The angiotensin K_m values were approx. 7 mM for pp60^{c-src} from both cell types. At the near-saturating concentration of ATP used in these experiments, the V_{max} for pp60^{c-src} from neuroblastoma cells was approx. 15-fold greater than that of pp60^{c-src} from fibroblasts (Table 1), in accord with the increased V_{max} of pp60^{c-src} from Neuro-2A cells observed at the near-saturating concentration of angiotensin I (20 mM) used in the first experiment.

The K_m values for pp60^{c-src} from Neuro-2A cells and fibroblasts were similar to the K_m values for ATP (25 \pm 4 μ M) and angiotensin I (6.6 \pm 0.5 mM) exhibited by the viral form of the enzyme (pp60^{v-src}) expressed in the Rous-sarcoma-virus-transformed cell line RR1022, which was also determined with the use of monoclonal antibody 327 in immune-complex protein kinase assays. It was not possible to compare the V_{max} values for pp60^{v-src} with those of pp60^{c-src} because monoclonal antibody 327 did not efficiently recognize pp60^{v-src} on Western immunoblots of RR1022-cell extracts. The amount of pp60^{v-src} protein could not be reliably compared with that of pp60^{c-src} in the neuroblastoma cells by metabolic labelling with [³⁵S]methionine owing to differential labelling of the viral and cellular forms of the enzyme.

DISCUSSION

By the use of an immune-complex protein kinase assay we show here that the pp60^{c-src} tyrosine kinase in the mouse neuroblastoma cell line Neuro-2A has a 7–15-fold higher V_{max} than the fibroblast enzyme, with only minor differences in K_m values for ATP and the phospho-acceptor substrate. These results indicate that the pp60^{c-src} kinase in neuroblastoma cells has a higher turnover number, which would enable it to exhibit enhanced catalytic activity even at the saturating concentrations of ATP found intracellularly.

The greater k_{cat} of the neuroblastoma enzyme appears to be a general feature of pp60^{c-src} in cells of neuronal origin. The pp60^{c-src} kinase from primary cultures of rat cortical neurons exhibited 6–12-fold greater specific activity than the enzyme in rat astrocyte cultures [9]. pp60^{c-src} in human neuroblastoma cell lines shows a 20–40-fold increase in specific activity compared with the enzyme in human glioblastoma cells or fibroblasts [13]. Because these studies were carried out in immune-complex assays at a single subsaturating concentration of ATP-Mg²⁺ (20 μ M [13]; 5 μ M [9]), it was not possible to predict whether differences in specific activity would persist at the saturating concentrations of ATP that are present in cells (2–3 mM) [28]. Our results showing that pp60^{c-src} from neuroblastoma Neuro-2A cells has an increased V_{max} at near-saturating concentrations of ATP suggests that substrate may be turned over to product at a greater rate by pp60^{c-src} in neuroblastoma cells than by pp60^{c-src} in fibroblasts. An increased V_{max} (5–20-fold) has also been reported for pp60^{c-src} bound to polyoma middle T antigen [27].

The 7–15-fold increased V_{max} of pp60^{c-src} kinase in the Neuro-2A neuroblastoma cell line compared with pp60^{c-src} in NIH(pMcsrc/cos)A fibroblasts depends on the relative amounts of pp60^{c-src} protein measured. The difference in amount of pp60^{c-src} protein in the two cell lines was found to vary from 10- to 20-fold, as determined by immunoblotting with monoclonal antibody 327. Immunoblotting with a different monoclonal antibody, AB src.1, showed 10–26-fold higher concentrations of pp60^{c-src} in the fibroblast cell line (results not shown). Thus inaccuracy in measurement of pp60^{c-src} protein in immunoblotting could decrease the observed differences in V_{max} by 2.6-fold at most, in which case the neuroblastoma enzyme would still exhibit a greater k_{cat} . The results reported here may be in error to the extent that the antibodies differentially recognize pp60^{c-src} from

the two cell lines on immunoblots. However, they are in accord with increased activity of the neuronal form of the enzyme reported by others using [³⁵S]methionine metabolic labelling to measure pp60^{c-src} protein. Differences in metabolic labelling of the Neuro-2A and NIH(pMcsrc/cos)A cell lines with [³⁵S]methionine and lack of a radioimmunoassay precluded measurement of pp60^{c-src} protein by an alternative method.

We do not know the nature of the modification responsible for the greater k_{cat} of pp60^{c-src} in neuroblastoma Neuro-2A cells. *N*-Terminal tyrosine phosphorylation within the pp60^{c-src} molecule has been observed in human neuroblastoma cells [13], and an unidentified *N*-terminal modification of pp60^{c-src} not due to tyrosine phosphorylation, that may be due to alternative splicing, has been observed in neuronal cultures [9]. However, in neither case has the modification been shown to contribute to the activity of the kinase. Several studies of pp60^{c-src} from mouse fibroblasts have demonstrated a correlation between dephosphorylation of tyrosine-527, usually accompanied by increased phosphorylation of tyrosine-416, and greater pp60^{c-src} protein kinase activity [29–33]. Cooper & King [29] observed that dephosphorylation of pp60^{c-src} by phosphatase treatment *in vitro* caused a 10–20-fold increase in pp60^{c-src} protein kinase activity that was attributable to a decreased K_m for ATP as well as an increased V_{max} . Similar *C*-terminal phosphorylations may play a role in the regulation of pp60^{c-src} protein kinase activity in Neuro-2A cells.

The implications of the results reported here are limited by the nature of the immune-complex protein kinase assay, necessitated by the lack of purified pp60^{c-src}. However; the ATP K_m value for pp60^{c-src} from RR1022 cells (25 μ M) obtained in the immune-complex protein kinase assay with monoclonal antibody 327 approximated a reported ATP K_m value (50 μ M) obtained with purified pp60^{v-src} assayed in solution with [Val⁵]-angiotensin II as phospho-acceptor substrate [34]. This indicates that the kinase activity of pp60^{c-src} in the immune complex may not be affected by binding to the monoclonal antibody.

The K_m values for angiotensin I for pp60^{c-src} from neuroblastoma Neuro-2A cells and fibroblasts were the same, indicating that the phospho-acceptor sites of the two forms of the enzyme were similar. It is possible that differences at the phospho-acceptor site exist that were not revealed by K_m values for the peptide substrate, and that differences in the K_m might be found with phospho-acceptor substrates other than angiotensin I. However, in lieu of a physiological substrate, angiotensin I is suitable for kinetic determinations, because it has a single tyrosine residue. *In vitro* protein substrates such as α -casein are likely to have multiple tyrosine residues that can be phosphorylated by pp60^{c-src}, rendering interpretation of kinetic data difficult. Nonetheless, it will be important to confirm the results presented here with natural cellular substrates, once they are identified, and also to determine if activation of pp60^{c-src} occurs in neuroblastoma Neuro-2A cells *in vivo*.

The physiological role of the enhanced catalytic efficiency of pp60^{c-src} kinase in neuroblastoma Neuro-2A cells is not known. The greater k_{cat} of pp60^{c-src} in Neuro-2A cells does not appear to involve a genetic alteration of *c-src* that is responsible for transformation of the neuroblastoma cells. Transfection of NIH 3T3 cells with

Neuro-2A genomic DNA resulted in foci of transformed cells whose DNA could be used in subsequent transfections to obtain secondary foci, indicating the presence of a transforming gene (D. W. Fults & P. F. Maness, unpublished work). DNA isolated from these secondary foci did not contain multiple copies of the *c-src* gene when analysed by Southern blotting. Moreover, extracts of transformed cells from the secondary foci did not exhibit elevated concentrations of pp60^{c-src} on Western blots or increased pp60^{c-src} kinase activity in immune-complex protein kinase assays, suggesting that the transforming gene of Neuro-2A cells is not the *c-src* gene.

The greater k_{cat} of pp60^{c-src} kinase from neuroblastoma cells appears instead to be a normal characteristic of neuronal cells. The expression of pp60^{c-src} in developing neurons of the neural retina and cerebellum is maximal during the period of neurite outgrowth, and immunoreactivity is concentrated in process-rich regions [6,7]. In addition, the ability of pp60^{v-src} to induce neurite outgrowth in PC12 pheochromocytoma cells implicates tyrosine kinase activity in process formation and neuronal differentiation [35]. The increased catalytic efficiency of pp60^{c-src} protein kinase in neuroblastoma cells may therefore be a manifestation of its activity in immature neuronal cells, which could involve neurite extension or some other aspect of neuronal differentiation.

This work was supported by a Public Health Service award (HD20406-01) from the National Institute of Child Health and Human Development. We thank Dr. Joan Brugge for monoclonal antibody 327 and Dr. David Shalloway for the NIH(pMcsrc/cos)A cell line. The excellent work of David Hillsgrove in the initial studies for this project is gratefully acknowledged. P. F. M. is a recipient of an N.I.H. Career Development Award.

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Received 20 January 1987/15 June 1987; accepted 17 August 1987