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Tyrosine Tubulin Kinases in Particulate Fractions from Rat Cerebral Cortex

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ABSTRACT

Tyrosine tubulin kinase activity in the particulate fractions from rat cerebral cortex was quantitatively solubilized and separated into two active peaks (kinase I and kinase II) by Sephacryl S-300 gel-filtration in the presence of 0.2% Nonidet P-40. Kinases I and II were each resolved into 5 active peaks (I-1→5 and II-1→5) by casein-Sepharose column chromatography. The molecular weights of these kinases were estimated from the $s_{20,w}$ values to be 59,000—65,000. Tyrosine-glutamate (1:4) copolymers were also substrates for the enzymes. About 60% of the copolymers kinase activity in I-3, I-4, II-3 and II-4 were immunoprecipitable with saturating amount of monoclonal antibody (MAb 327) that recognizes pp60$^{c\text{-}src}$. The Km values of II-3 and II-4 for tubulin were nearly 10 times higher than those of I-3 and I-4. However, the Km values of the four kinases for the copolymers were not so significantly different. In comparison with the II-3 and II-4 kinase fractions, I-3 and I-4 fractions showed 2 and 10 times higher activity ratios with tubulin and the IgG heavy chain of MAb 327 relative to the copolymers kinase activity. Incubation of the immunocomplexes with ATP at 0°C for 10 min resulted in the autophosphorylation of a 60kDa protein in I-3 and I-4 but not in II-3 and II-4.

Since tyrosine protein kinase activity was first found to reside in the oncogene product (pp60$^{v\text{-}src}$) of Rous sarcoma virus[17] and subsequently in receptors of various growth factors such as epidermal growth factor (EGF)[41], insulin[19], platelet-derived growth factor[13], insulin-like growth factor I[18,30] and colony stimulating factor-1[34], it has been suggested that the phosphorylation of tryosine is associated with cell transformation or cell proliferation. However, a survey of tyrosine protein kinase activity in various tissues and blood cells in mammals and in sea urchin embryos[13,35] has revealed that such activity is abundant in membrane fractions of spleen[38], brain[12], thymus[24] and even unnucleated erythrocytes[26] and platelets[14]. We have also shown that endogenous proteins are phosphorylated *in vitro* on tyrosine residues to a larger extent in mouse liver membranes than in MH134 hepatoma and Ehrlich ascites tumor cell membranes[42]. These findings suggest that tyrosine protein kinase activity does not necessarily correlate with the proliferative activity of the cell but rather has a relation to the differentiated functions of the cell.

During our search for the exogenous substrates of the membrane tyrosine protein kinases, we found that brain tubulin was preferentially phosphorylated at tyrosine residues by the membrane fractions[48]. The partially purified tyrosine protein kinases from membrane fractions of mouse liver and Ehrlich ascites tumor cell phosphorylated tubulin stoichiometrically with the Km values for tubulin reasonably lower than the estimated intracellular concentrations of tubulin, suggesting that tubulin might be one of the physiological sub-

strates of the tyrosine protein kinases[48]. In our investigation of the possible role of the phosphorylation of tyrosine residue in tubulin, we thought that rat brain would be the most appropriate source for tyrosine tubulin kinases, since this tissue has been known to be rich not only in tubulin, but also in tyrosine protein kinases[12].

In this paper I describe the existence of at least ten separable peaks showing tyrosine tubulin kinase activity in particulate fractions from rat cerebral cortex. Although all of the kinases had molecular weights of about 60,000, they were separated into two groups, kinase I and kinase II, by gel-filtration in the presence of 0.2% Nonidet P-40 (NP-40). Four of them were immunoprecipitable with monoclonal antibody that recognizes $pp60^{c\text{-}src}$. The immunoprecipitable enzymes of the kinase I group were clearly distinguishable from those of the kinase II group by their catalytic properties.

MATERIALS AND METHODS

Materials

Tubulin was purified from bovine brain[20] and heated for 3 min at 60°C to inactivate contaminating protein kinases. Casein-Sepharose 4B was prepared as described[40] except that casein (15 mg/g Sepharose 4B gel) was used as ligand. Wheat germ agglutinin (WGA)-agarose was obtained from Hohnen Oil Company. EGF from mouse submaxillary gland was purchased from Toyobo. Bovine insulin, phenylmethylsulfonyl fluoride (PMSF), tyrosine-glutamate (1:4) copolymers (Mr = 36,000) and protein A-Sepharose CL-4B were obtained from Sigma. Dithiothreitol (DTT) was obtained from Boehringer-Manheim. A monoclonal antibody MAb 327, which recognizes $pp60^{c\text{-}src}$ [21], was purified by DEAE Affi-gel blue (Bio-Rad) from the ascites fluid from hybridoma 327. Chymostatin, pepstatin, leupeptin and antipain were obtained from Peptide Institute Inc. Other chemicals were obtained from various commercial sources.

Buffer solutions

All the buffer solutions contained 0.5 mM PMSF, 0.3 μg/ml chymostatin, 0.3 μg/ml pepstatin, 2 μg/ml antipain, 1 μg/ml leupeptin and 1 mM benzamidine as protease inhibitors. Buffer A contained 10 mM Hepes, pH 7.4, 11% (w/v) sucrose, 1 mM EDTA, 0.5 mM DTT and protease inhibitors; Buffer B contained 20 mM Hepes, pH 7.4, 5% sucrose, 0.2% (v/v) NP-40, 1 mM EDTA, 0.5 mM DTT and protease inhibitors; Buffer C contained 50 mM Hepes, pH 7.4, 0.15 M NaCl, 0.2% NP-40, 5% sucrose, 0.5 mM DTT and protease inhibitors.

Assay for tyrosine protein kinase

Tyrosine protein kinase activity was assayed in the standard assay mixture (60 μl) containing 20 mM Hepes, pH 7.4, 3 mM $MnCl_2$, 10 μM $ZnCl_2$, 30 μM $NaVO_3$, 0.5 mM DTT, 0.2% NP-40, 1 mg/ml (10 μM) tubulin or 0.05 mg/ml tyrosine-glutamate copolymers, 7.5 μM [γ-^{32}P]ATP (0.6-3.0 × 10^3 cpm/pmol) and enzyme preparation. After incubating for 10 min at 30°C, a 50 μl aliquot was spotted on a Whatman 3 MM paper, and further processed[3]. Since tubulin was also phosphorylated at serine and threonine residues by serine and threonine protein kinases contaminated in enzyme preparations, phosphorylation of tyrosine residues in tubulin was estimated by the alkali-treatment of the filter paper[43]. The estimated value was comparable to that obtained by phosphoamino acid analysis[48] of phosphorylated tubulin which was isolated by SDS-polyacrylamide gel electrophoresis. The ^{32}P counts were measured by Cerenkov radiation[4]. A control value without the substrates was subtracted from the complete value.

One unit of the enzyme activity was defined as the amount of enzyme which transferred 1 pmol of phosphate from ATP to the substrate per min.

Preparation of tyrosine protein kinases

Unless otherwise stated, all procedures were carried out at 0—4°C. Seven male Wistar albino rats (200—250 g) were starved overnight, and the cerebral cortex (8.0 g) was homogenized with 32 ml of Buffer A in a Teflon-glass homogenizer (4 strokes). The homogenate was centrifuged at 800 × g for 10 min. The pellet was washed once with 16 ml of Buffer A. The washings and the original supernatant were combined and centrifuged at 2,500 × g for 10 min. The pellet was washed once with 16 ml of Buffer A. The washings and the original supernatant were combined and centrifuged at 100,000 × g for 60 min. The pellet was suspended in 7 ml of Buffer A (Particulate fractions). The particulate fractions (145 mg protein) were shaken gently at 0°C for 60

min in 10 ml of Buffer A containing 2% NP-40 and 0.5 M NaCl. After centrifugation at 200,000 × g for 60 min, the supernatant (NP-40 extract) was applied to a Sephacryl S-300 column (80 × 2.5 cm) equilibrated with Buffer B containing 0.2 M NaCl. Elution was performed upward with the same buffer at a flow rate of 35 ml/hr and fractions of 4 ml each were collected. Tyrosine protein kinase activity was resolved into two peaks (Fig. 1). These two active peaks were designated kinase I and kinase II. Kinase I fractions (Fig. 1) were pooled (Sephacryl S-300 (I) fraction) and 15% (w/v) of sodium cholate was added to final 0.7%, followed by saturated ammonium sulfate solution (pH 7.4) to final 45% saturation. After standing for 2 hr, the precipitate was collected by centrifugation at 125,000 × g for 60 min and suspended in 10 ml of Buffer B containing 0.2 M NaCl and 0.7% sodium cholate. The suspension was centrifuged at 41,000 × g for 20 min. To the supernatant (Concentration fraction) was added 12% NP-40 to final 2%. This was then applied to a Sephacryl S-300 column (80 × 2.5 cm) equilibrated with Buffer B containing 0.2 M NaCl. Kinase II fractions (Fig. 1) were pooled (Sephacryl S-300 (I) fraction) and concentrated to 10 ml by ultra-filtration with an Amicon YM-10 membrane, and centrifuged at 41,000 × g for 20 min. The supernatant (Concentration fraction) was adjusted to 2% with 12% NP-40 and applied to a Sephacryl S-300 column as described for the kinase I fractions. Elution of these columns was performed upward with the same buffer at a flow rate of 35 ml/hr and fractions of 4 ml each were collected. Fractions of kinase I and II (Fig. 2) were separately pooled (Sephacryl S-300 (II) fraction). One third volume (v/v) of 140 mM Hepes, pH 7.4, 0.2% NP-40, 3 mM $MnCl_2$, 5% sucrose, 0.5 mM DTT and protease inhibitors (see *Buffers*) were added and the fractions were applied at a flow rate of 6 ml/hr to a WGA-agarose column (0.1 ml per 200 units of tyrosine tubulin kinase, 0.7 cm diameter) equilibrated with Buffer C. The enzyme solutions were recycled twice at a flow rate of 20 ml/hr through each column; the final flow-through was saved, and the columns were washed with 4 column volumes of Buffer C. The washing eluate was combined with the final flow-through (WGA-agarose fraction) and dialyzed against 25 volumes of Buffer B for 3 hr. The kinase I fraction (3,632 units, 13 mg protein) was divided into two parts, and kinase II fraction and the two parts of kinase I fraction were separately applied at a flow rate of 10 ml/hr to a casein-Sepharose column (7.5 × 1.6 cm) equilibrated with Buffer B. After the column was washed with 4 column volumes of Buffer B, the enzyme was eluted at a flow rate of 20 ml/hr with a 400 ml linear 0—0.4 M NaCl gradient in Buffer B. Fractions of 4 ml each were collected. Kinase I was resolved into five activity peaks which were designated I-1, I-2, I-3, I-4 and I-5 as shown in Fig. 4A. Kinase II was also resolved into five activity peaks which were designated II-1, II-2, II-3, II-4 and II-5 as shown in Fig. 4B. Fractions of I-3, I-4, I-5, II-3 and II-4 (Fig. 4) were separately pooled and dialyzed against 10 volumes of Buffer B for 1 hr. Fractions of I-3 (476 units, 1.98 mg protein), I-4 (646 units, 0.83 mg protein), I-5 (283 units, 0.47 mg protein), II-3 (24 units, 0.40 mg protein) and II-4 (92 units, 0.40 mg protein) were separately applied at a flow rate of 20 ml/hr to a casein-Sepharose column (3 × 1.1 cm for I-3, I-4, I-5 and II-4, 3 × 0.7 cm for II-3) equilibrated with Buffer B containing 0.15 M NaCl (I-4 and I-5), 0.05 M NaCl (II-3) or 0.1 M NaCl (I-3 and II-4). After the column was washed with 4 column volumes of equilibration buffers, the enzyme was eluted with a 80 ml (30 ml for II-3) linear 0.1—0.3 M (I-3), 0.15—0.35 M (I-4 and I-5), 0.05—0.2 M (II-3) or 0.1—0.25 M (II-4) NaCl gradient in Buffer B. The flow rate was 6 ml/hr (2 ml/hr for II-3) and 1 ml fractions (0.6 ml fractions for II-3) were collected. Peak fractions were pooled and used as final I-3, I-4, I-5, II-3 and II-4 preparations. The enzymes could be stored at −80°C for three months without significant loss of activity. A summary of the purification is presented in Tables 1 and 2.

Analysis of autophosphorylation

The phosphorylation reactions (60 μl) were stopped by adding 20 μl of 40 mM Tris-HCl, pH 7.8, 12% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 0.07% (w/v) bromophenol blue, and 8% (v/v) 2-mercaptoethanol (SDS buffer) and were then boiled for 3 min. Fifty μl of sample was applied to SDS-polyacrylamide gel (10% gel) electrophoresis[31]. The gels were stained[45], dried, treated with alkali[5], and au-

Table 1. Preparation of tyrosine protein kinases from rat cerebral cortex.
The enzymes were purified from 8.0 g of rat cerebral cortex. Purification and assay with tubulin as substrate were described under Materials and Methods.

Step	Protein	Total activity	Specific activity	Yield
	(mg)	(units)	(units/mg)	(%)
1. Particulate fractions	145	6,960	48	100
2. NP-40 extract	117	7,090	61	102
3. Sephacryl S-300 (I)				
Kinase I	74	5,580	76	80.2
Kinase II	29	1,080	38	15.5
4. Concentration				
Kinase I	29	5,060	175	72.7
Kinase II	28	1,140	41	16.4
5. Sephacryl S-300 (II)				
Kinase I	14	4,520	314	64.9
Kinase II	13	456	35	6.6
6. WGA-agarose				
Kinase I	13	4,390	341	63.1
Kinase II	11	441	39	6.3

Table 2. Resolution of kinases I and II subgroups by casein-Sepharose.
Kinases I and II (step 6 in Table 1) were chromatographed on casein-Sepharose and assayed with tubulin as substrate as described under Materials and Methods.

Enzyme	Protein	Total activity	Specific activity	Yield
	(mg)	(units)	(units/mg)	(%)
I-1	2.27	18	8	0.3
I-2	0.95	95	100	1.4
I-3	0.26	160	608	2.3
I-4	0.13	195	1,450	2.8
I-5	0.14	139	994	2.0
II-1	6.03	22	4	0.3
II-2	1.02	40	39	0.6
II-3	0.13	10	77	0.1
II-4	0.10	35	350	0.5
II-5	0.10	25	252	0.4

toradiographed using X ray films (Fuji RX) with intensifying screens (Fuji Hi-Screen) at −80°C.

Immunoprecipitation with anti-pp60^{src} IgG

Immobilized IgGs were prepared as described by Wong and Goldberg[47] except that 10 μl of MAb 327 (4.8 mg/ml) was immobilized in 20 μl of protein A-Sepharose (a 1:1 suspension in 50 mM potassium phosphate buffer, pH 7.5). Forty μl of enzyme solution was added to immobilized IgGs and incubated at 0°C for 2 hr with resuspension by gentle tapping every 15 min. Enzyme mixtures were then centrifuged at 14,000 × g for 5 min at 4°C and aliquots of supernatants were removed to assay tyrosine protein kinase activities. The pelleted Sepharose beads were washed three times with 0.5 ml of Buffer B and added 50 μl of the standard assay mixture containing 0.05 mg/ml tyrosine-glutamate copolymers. After 10 min at 30°C, the reactions were terminated by the addition of 10 μl of 210 mM EDTA, and the mixtures were centrifuged at 14,000 × g for 2 min at 4°C. Fifty μl aliquots of supernatants was spotted on a Whatman 3 MM paper and followed as described above.

Other determinations

The $s_{20,w}$ values were determined by sucrose density gradient centrifugation[23] except that a linear gradient of 5 to 20% (w/v) sucrose in Buffer B containing 0.2 M NaCl was employed. The molecular weights were estimated from $s_{20,w}$ values[10]. Protein concentration was determined by the method of Lowry et al[22].

RESULTS

Solubilization and Resolution of Kinases I and II in the Cerebral Cortex-When tyrosine protein kinase activity was measured with tubulin or tyrosine-glutamate (1:4) copolymers as substrate, the specific activities in the particulate fractions from rat cerebellum, medulla oblongata, hypothalamus, striatum, midbrain and cerebral cortex were similar and more than 3 times higher than those in the soluble fractions (data not shown). More than 60% of the total kinase activities were present in the particulate fractions of each region, the highest being in the

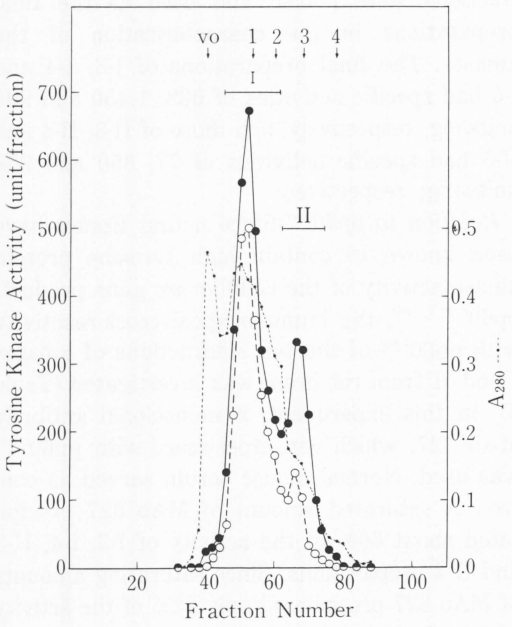

Fig. 1. Resolution of kinases I and II by Sephacryl S-300 gel filtration. Gel filtration and measurement of tyrosine protein kinase activity toward either tubulin (○) or tyrosine-glutamate copolymers (●) were performed as described under Materials and Methods. The arrows indicate the elution positions of blue dextran (Vo), ferritin (1), catalase (2), ovalbumin (3) and cytochrome *c* (4).

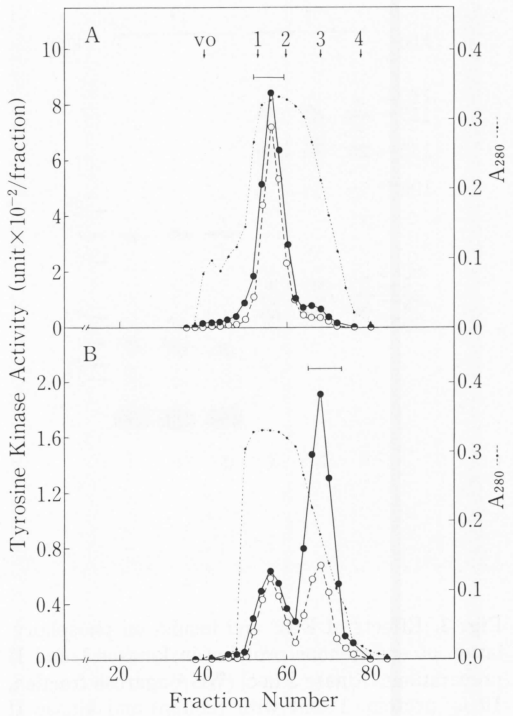

Fig. 2. Rechromatography of kinases I and II on Sephacryl S-300. Gel filtration of kinase I (A) and kinase II (B) and measurement of tyrosine protein kinase activity toward either tubulin (○) or tyrosine-glutamate copolymers (●) were performed as described under the legend to Fig. 1.

cortex (data not shown). Therefore, the particulate fractions of cerebral cortex were chosen as a source of tyrosine protein kinase. Tyrosine protein kinase activities toward tubulin and tyrosine-glutamate copolymers were quantitatively solubilized from the particulate fractions of the cerebral cortex with a buffer containing 2% NP-40 and 0.5 M NaCl. If 0.5 M NaCl was omitted from the solubilization buffer, only about 60% of the activities were solubilized.

The solubilized tyrosine protein kinase activities were resolved into two peak fractions by gel filtration on Sephacryl S-300 in the presence of 0.2% NP-40 (Fig. 1). The peak fractions were separately pooled and designated kinase I and kinase II, respectively, according to the order of elution. To eliminate cross-contamination, the pooled fractions of kinase I and kinase II were concentrated and subjected separately to rechromatography on the same Sephacryl S-300 column (Fig. 2). The active fractions (Fig. 2) were pooled and used as kinases I and II fractions.

Relationship to Insulin and EGF Receptors- When kinases I and II were applied to WGA-agarose column chromatography under conditions where insulin and EGF receptors were completely adsorbed[15,16], more than 96% of the tyrosine tubulin kinase activity of kinases I and II passed through the column (Table 1). Furthermore, alkali-resistant autophosphorylation of endogenous proteins in the flow-through preparations of kinases I and II were not influenced by preincubation of the preparations with either insulin or EGF (Fig. 3). The 95kDa β-subunit of the insulin receptor and the 170kDa EGF receptor, which are known to be autophosphorylated in the presence of growth factor at tyrosine residues[19,41], were not detected among the endogenously phosphorylated proteins (Fig. 3), indicating that the WGA-agarose fractions of kinases I and II were free of insulin and EGF receptors.

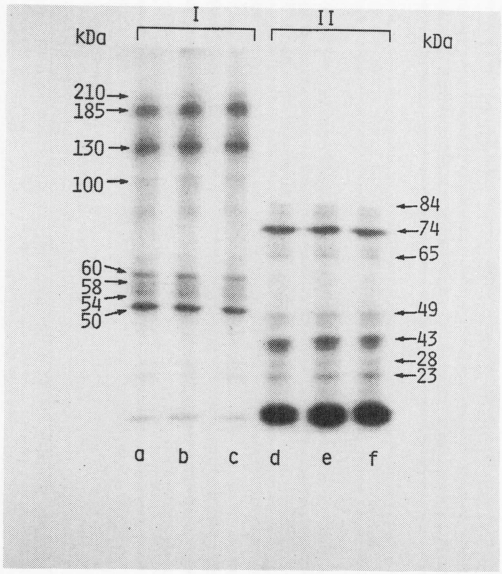

Fig. 3. Effects of EGF and insulin on phosphorylation of endogenous proteins in kinases I and II preparations. Kinase I (a-c) (WGA-agarose fraction, 10 μg protein, 1 unit with tubulin) and kinase II (d-f) (WGA-agarose fraction, 24 μg protein, 0.8 unit with tubulin) were incubated separately with 15 μM [γ-^{32}P]ATP (10^4 cpm/pmol) in the absence (a, d) or presence of 0.22 μM EGF (b, e) or 1 μM insulin (c, f) at 0°C for 10 min in 60 μl of the standard assay mixture except that exogenous substrates were omitted. Before adding 10 μl of [γ-^{32}P]ATP solution, the rest of the reaction mixture was preincubated at 0°C for 30 min. Analysis of autophosphorylation by SDS-polyacrylamide gel electrophoresis was performed as described under Materials and Methods.

Casein-Sepharose Column Chromatography of Kinases I and II-The flow through fractions of kinases I and II from the WGA-agarose columns were further purified by chromatography on casein-Sepharose columns (Fig. 4). Kinases I and II were resolved into five peaks, respectively. These active peaks were numbered in the order of elution and designated I-1, I-2, I-3, I-4 and I-5 for the activity peaks of kinase I (Fig. 4A) and II-1, II-2, II-3, II-4 and II-5 for the activity peaks of kinase II (Fig. 4B). At this purification stage, about 80% of the total tyrosine tubulin kinase activity resided in peaks I-3, I-4 and I-5 (Table 2). Peaks I-3, I-4, I-5, II-3 and II-4 were rechromatographed on casein-Sepharose in order to improve the resolution and eluted with a linear NaCl concentration gradient. The peak fractions were pooled and used as the final preparations in the characterization of the kinases. The final preparations of I-3, I-4 and I-5 had specific activities of 608, 1,450 and 994 units/mg, respectively, and those of II-3, II-4 and II-5 had specific activities of 77, 350 and 252 units/mg, respectively.

Relation to pp60$^{c\text{-}src}$-Since neural tissues have been known to contain high tyrosine protein kinase activity of the cellular *src* gene product, pp60$^{c\text{-}src}$ [1,8,36], the immunological cross-reactivity with pp60$^{c\text{-}src}$ of the ten subfractions of kinases I and II from rat brain was investigated (Table 3). In this experiment, a monoclonal antibody MAb 327, which can cross-react with pp60$^{c\text{-}src}$, was used. Normal mouse serum served as control. A saturated amount of MAb 327 precipitated about 60% of the activity of I-3, I-4, II-3 and II-4 preparations. Since saturating amounts of MAb 327 precipitated only 22% of the activity of I-5, the precipitated activity is suggested to result from the cross-contamination of I-4 in I-5. Other enzyme fractions showed essentially no immunological cross-reactivity with pp60$^{c\text{-}src}$.

Molecular Parameters-The apparent Stokes radii of tyrosine protein kinases I and II were estimated to be 6.68 ± 0.40 nm and 2.76 ± 0.37 nm by gel filtration on Sephacryl S-300 in the presence of 0.2% NP-40 (Fig. 2). However, the apparent sedimentation constants (s_{20},w) of the ten subfractions of kinases I and II as determined by sucrose density gradient centrifugation ranged from 3.4 to 3.8S (Table 4). From the s_{20},w values, the molecular weights of these kinases were estimated to be in the range of 59,000 to 65,000. The apparent molecular weights of I-3, I-4, II-3 and II-4 were estimated to be 60,000, 61,000, 61,000 and 62,000, respectively, which approximate to a reasonable degree the molecular weight of pp60$^{c\text{-}src}$.

Autophosphorylation-Since pp60$^{c\text{-}src}$ is autophosphorylated at a tyrosine residue *in vitro* [35], I attempted to detect pp60$^{c\text{-}src}$ immunologically after labeling the kinase with [γ-^{32}P]ATP by SDS-polyacrylamide gel electrophoresis, alkali-treatment and autoradiography (Fig. 5). The 60kDa protein bands which contained alkali-resistant phosphate were indeed detected in the immunoprecipitates of I-3 and I-4 but not in the immunoprecipitates of the II-3 and II-4 preparations (Fig. 5). Although the

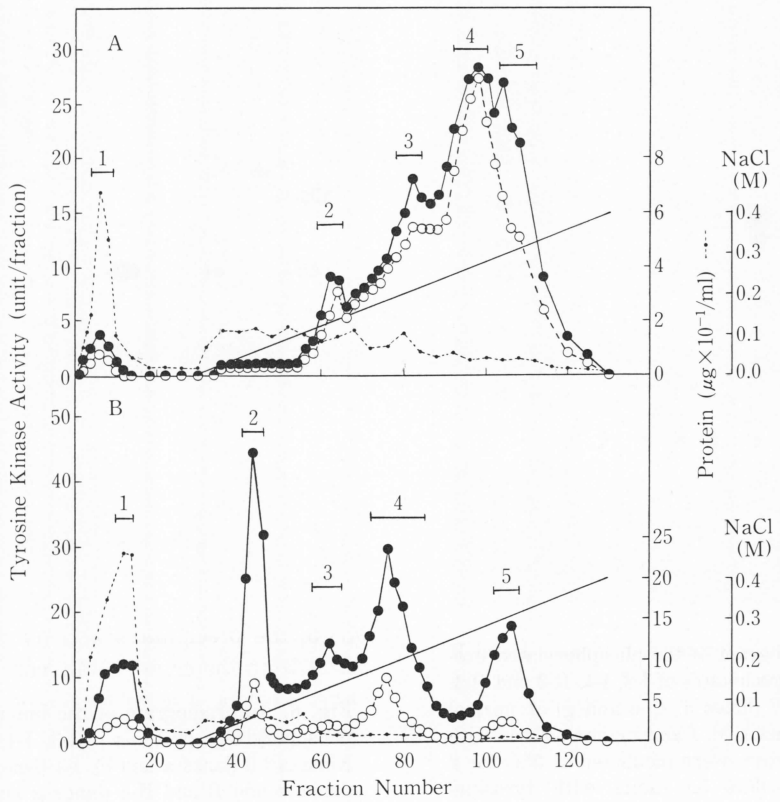

Fig. 4. Casein-Sepharose column chromatography of kinases I and II. Kinase I (A) and kinase II (B) were chromatographed and assayed as described under Materials and Methods, with tubulin (○) or tyrosine-glutamate copolymers (•) as substrate.

Table 3. Immunoprecipitation with anti-pp60^{src} IgG of kinases I and II subgroups. Immunoprecipitation and assay for the tyrosine protein kinase activity with tyrosine-glutamate copolymers were performed as described under Materials and Methods. Values are the percentage activity compared to normal mouse serum.

Enzyme	Kinase activity	
	Supernatant	Precipitate
	(%)	
I-1	101	1
I-2	100	4
I-3	42	58
I-4	34	66
I-5	78	22
II-1	101	0
II-2	102	1
II-3	36	61
II-4	24	64
II-5	94	5

Table 4. Molecular parameters of kinases I and II subgroups.
The $s_{20,w}$ values and the molecular weights were determined as described under Materials and Methods. The average values and deviations for two separate experiments are given.

Enzyme	$s_{20,w}$	Molecular weight ($\times 10^{-3}$)
	(S)	
I-1	3.83 ± 0.03	65 ± 0
I-2	3.40 ± 0.08	60 ± 1
I-3	3.43 ± 0.09	60 ± 1
I-4	3.46 ± 0.11	61 ± 2
I-5	3.73 ± 0.07	64 ± 1
II-1	3.45 ± 0.06	61 ± 1
II-2	3.38 ± 0.12	59 ± 2
II-3	3.46 ± 0.12	61 ± 2
II-4	3.57 ± 0.01	62 ± 0
II-5	3.69 ± 0.02	64 ± 1

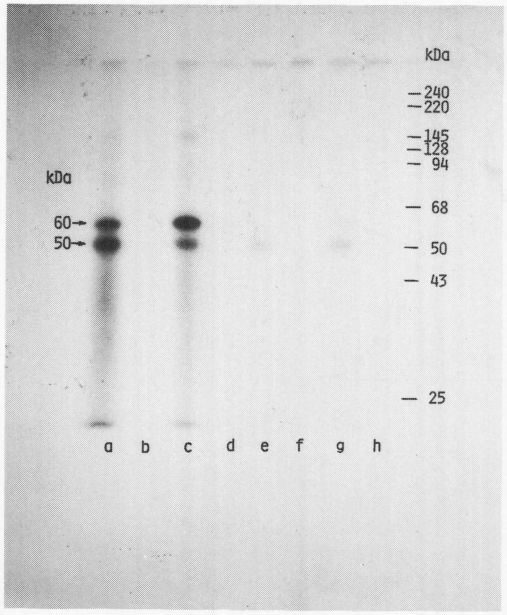

Fig. 5. Autoradiogram of the phosphorylated proteins in immunoprecipitates of I-3, I-4, II-3 and II-4. IgGs of MAb 327 (lanes a, c, e and g) or normal mouse serum (lanes b, d, f and h), immobilized with protein A-Sepharose, were incubated at 0°C for 2 hr with 80 μl (3.5~5.6 units with tyrosine-glutamate copolymers) of either I-3 (lanes a and b), I-4 (lanes c and d), II-3 (lanes e and f) or II-4 (lanes g and h). The immunoprecipitates (about 2 units with tyrosine-glutamate copolymers) were incubated with [γ-^{32}P]ATP (1.3×10^5 cpm/pmol) at 0°C for 10 min in 60 μl of the standard assay mixture. Analysis of autophosphorylation was performed as described under Materials and Methods.

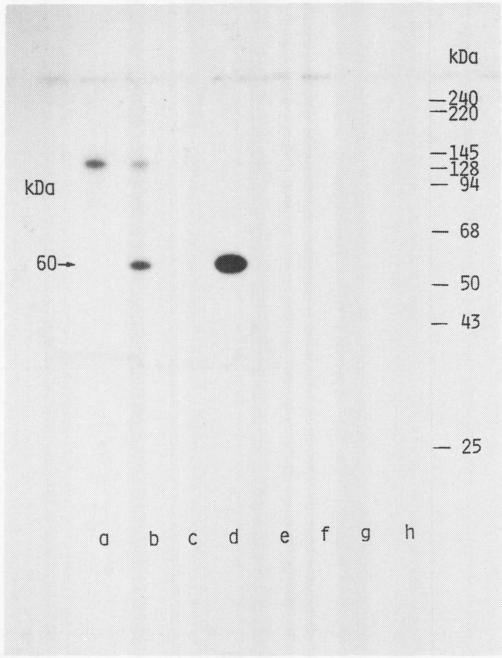

Fig. 6. Autoradiogram of the immunoprecipitable phosphorylated proteins in I-3, I-4, II-3 and II-4. Kinases I-3 (lanes a and b), I-4 (lanes c and d), II-3 (lanes e and f) and II-4 (lanes g and h) (2.9 units with tyrosine-glutamate copolymers) were incubated with [γ-^{32}P]ATP (6.4×10^4 cpm/pmol) at 0°C for 10 min in 60 μl of the standard assay mixture. The reactions were terminated by the addition of 10 μl of 210 mM EDTA. Sixty μl aliquots were incubated at 0°C for 2 hr with IgGs of MAb 327 (lanes b, d, f and h) or normal mouse serum (lanes a, c, e and g), immobilized with protein A-Sepharose. The phosphorylated proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described under Materials and Methods.

50kDa IgG heavy chains in the precipitates containing alkali-resistant phosphate, about 10 times more phosphate was incorporated into the 50kDa protein with I-3 and I-4 than with II-3 and II-4 (Fig. 5). The possible interference by MAb 327 of the autophosphorylation of II-3 and II-4, was eliminated by first incubating the II-3 and II-4 preparations with [γ-^{32}P]ATP and then immunoprecipitating with MAb 327, prior to gel electrophoresis (Fig. 6). The autophosphorylation of II-3 and II-4 was again not detected in the immunocomplexes under the conditions in which autophosphorylated I-3 and I-4 were detected. These results suggest the existence of two different states of pp60$^{c\text{-}src}$, one, illustrated by preparations I-3 and I-4 was readily autophosphorylated and the other, illustrated by fractions II-3 and II-4 was hardly autophosphorylated.

Kinetic Properties-The apparent Km values for tubulin were determined to be 1.1 μM and 0.71 μM with I-3 and I-4, and 9.2 μM and 6.1 μM with II-3 and II-4 (Table 5), indicating that I-3 and I-4 had a higher affinity for tubulin by nearly 10 fold than the II-3 and II-4. The apparent Km values for tyrosine-glutamate copolymers were determined to be 0.049 μM and 0.025 μM with I-3 and I-4, and 0.092 μM and 0.044 μM with II-3 and II-4 (Table 5). These data indicate that the differences in the Km values for the copolymers between I-3 and I-4, and II-3 and II-4 were less significant than those for tubulin.

Table 5. Kinetic properties of I-3, I-4, II-3 and II-4. Protein kinase activities with various concentration of substrates were measured with 0.05 unit of enzyme fractions under the standard assay condition. Km values were estimated by fitting the data to Michaelis-Menten equations using the method of least squares[32]. Km values are averages ± S.D. of more than three separate analyses.

Enzyme	Km values for		Vmax ratio
	Tubulin	Copolymers	Tubulin/Copolymers
	(μM)	(μM)	
I-3	1.09 ± 0.30	0.049 ± 0.006	0.986
I-3[a]	1.03 ± 0.18	0.042 ± 0.005	0.993
I-4	0.708 ± 0.257	0.025 ± 0.012	1.19
I-4[a]	0.928 ± 0.347	0.030 ± 0.008	1.06
II-3	9.17 ± 2.19	0.092 ± 0.011	0.670
II-3[a]	9.83 ± 3.07	0.073 ± 0.018	0.627
II-4	6.07 ± 1.49	0.044 ± 0.018	0.787
II-4[a]	7.30 ± 1.28	0.040 ± 0.008	0.410

[a]immunocomplexes with MAb 327 were used as enzyme preparations.

Comparison of the Vmax ratios with tubulin relative to tyrosine-glutamate copolymers of I-3, I-4, II-3 and II-4 revealed 2 times higher values for I-3 and I-4 than for II-3 and II-4.

Assuming that these fractions are equally active with tyrosine-glutamate copolymers as substrate, these data indicate that I-3 and I-4 are 2 times more active toward tubulin than II-3 and II-4.

DISCUSSION

The tyrosine protein kinase activity in particulate fractions from rat cerebral cortex was resolved into two peaks, namely kinase I and kinase II, by gel filtration on Sephacryl S-300 in the presence of 0.2% NP-40. After rechromatography of these two peaks, they eluted mainly with the same volumes needed for the first gel-filtration. These results indicate that the activity peaks of kinases I and II represent two different groups of enzymes and not two equilibrated forms of the same enzymes.

Kinases I and II were further resolved, respectively, into five peaks I-1→5 and II-1→5 on casein-Sepharose. Peaks I-3, I-4 and I-5 composed up to 80% of the total tyrosine tubulin kinase activity solubilized from the cortex particulate fractions.

The kinase activity in the ten peaks derived from kinases I and II sedimented at about the same rate during sucrose gradient centrifugation, with molecular weights estimated from the apparent $s_{20,w}$ values to be about 60,000 (Table 4). The immunocomplex of I-3 and I-4 with monoclonal antibody MAb 327 also produced a single 60kDa alkali-resistant phosphorylated band (Figs. 5 and 6). This antibody recognizes the common aminoterminal sequences of $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$ without interfering with its tyrosine protein kinase activity. Autophosphorylation of the I-5 preparation likewise yielded an alkali-resistant phosphoprotein with a molecular weight of about 60kDa (data not shown) on polyacrylamide gel electrophoresis.

Interestingly, decreasing the concentration of NP-40 from 0.2 to 0.05% during the Sephacryl S-300 chromatography caused a shift of the elution position of kinase I from higher to lower molecular weight (data not shown). A similar shift of the elution position on gel filtration of tyrosine protein kinase solubilized from plasma membrane fractions from rat liver and Ehrlich ascites tumor cells has been reported[48]. One possible interpretation of this observation is that in the presence of 0.2% NP-40, kinase I (but not kinase II), may bind to NP-40 molecules, probably forming micelles. As a result the kinase I elutes at a position corresponding to a higher molecular weight complex. Such an interaction between kinase I and NP-40 could be decreased when a lower concentration of NP-40 is used or when the kinase is in the presence of a high concentration of sucrose, such as during sucrose gradient centrifugation. It is also possible,

though less likely, that kinase I forms a complex with multiple proteins in the presence of 0.2% NP-40. Further work is necessary to distinguish between these possibilities.

About 60% of the tyrosine-glutamate copolymers kinase activity of I-3, I-4, II-3 and II-4 were immunoprecipitated with saturating amounts of MAb 327 (Table 3). The activity toward tubulin relative to tyrosine-glutamate copolymers of immunoprecipitable II-3 and II-4 was obviously lower than that of I-3 and I-4 (Table 5). Fractions II-3 and II-4 also showed much lower activity toward the IgG heavy chain of MAb 327 than fractions I-3 and I-4, whose activity toward tyrosine-glutamate copolymers was comparable to that of II-3 and II-4 (Fig. 5). Furthermore, the Km values of immunoprecipitable II-3 and II-4 for tubulin were 10 times higher than those of I-3 and I-4, while, the Km values of II-3 and II-4 for tyrosine-glutamate copolymers were only 2 times, or less, higher than those of I-3 and I-4 (Table 5). These data suggest the existence of active and inactive forms of $pp60^{c\text{-}src}$. Some of tyrosine protein kinases[27,39,46] including EGF receptor[2] and insulin receptor[29,49], are known to be activated by autophosphorylation. Since autophosphorylation of I-3 and I-4 but not II-3 and II-4 was observed in immunocomplexes of these kinases with MAb 327 (Figs. 5 and 6), the lower tyrosine tubulin kinase activity of II-3 and II-4 may be attributable to a lack of autophosphorylation. Tyrosine-glutamate copolymers are known to block the autophosphorylation of insulin receptor tyrosine kinase[25]. The copolymers may block the autophosphorylation of I-3 and I-4 and thus suppress tyrosine kinase activity. This may explain the smaller differences between kinase I subfractions and kinase II subfractions in their Km values for the copolymers and in their kinase activities toward the copolymers.

The tyrosine protein kinase activity of $pp60^{c\text{-}src}$ is known to be suppressed by the phosphorylation of Tyr-527 with concomitant failure to phosphorylate Tyr-416[6,7,9]. Since II-3 and II-4 were immunoprecipitated with MAb 327 and could not autophosphorylate, they might be $pp60^{c\text{-}src}$ which is phosphorylated at Tyr-527. On the other hand, I-3 and I-4 can autophosphorylate, and are assumed to be $pp60^{c\text{-}src}$, which is not phosphorylated at Tyr-527. The phosphorylation of Tyr-527 may counteract the hydrophobic interaction between Tyr-527 of the $pp60^{c\text{-}src}$ and NP-40. Thus, the binding to NP-40 of I-3 and I-4 (which are expected to be unphosphorylated at Tyr-527) is tighter than the binding to NP-40 of II-3 and II-4 (which are assumed to be phosphorylated at Tyr-527). These differences in the binding may underline the separation of kinase I and kinase II by gel filtration on Sephacryl S-300 in the presence of 0.2% NP-40.

Similar relationships are also suggested between kinase I and kinase II subfractions other than I-3, I-4, II-3 and II-4. For example, the Km values for tubulin of the kinase I subgroups were 10 times lower than those of the kinase II subgroups (data not shown). Also, while the 60kDa, 58kDa and 54kDa bands were autophosphorylated in the kinase I preparation, no radioactive bands were detected in this region with the kinase II preparation (Fig. 3).

It is unlikely that the other six tyrosine protein kinase peaks which could not cross-react with MAb 327 are degradative products of $pp60^{c\text{-}src}$, since several protease inhibitors (see *Buffers*) were present throughout the preparation of the enzymes. Furthermore, their $s_{20,w}$ values were similar to those of I-3, I-4, II-3 and II-4, and their estimated molecular weights were approximately 60,000. The existence of 60kDa tyrosine protein kinases other than $pp60^{c\text{-}src}$, such as products of cellular *yes* and *fyn* genes[33,37] also supports the notion that the multiple tyrosine protein kinases are not artifacts due to proteolytic degradation.

Some of the kinase I and kinase II subgroups may be interconvertible by phosphorylation and dephosphorylation of certain tyrosine residues of the kinase molecules. This tyrosine phosphorylation may be catalyzed by the other tyrosine protein kinases. There may be cascades involving tyrosine phosphorylation of enzymes to regulate activity during signal transduction through the membrane. Further studies are needed to clarify the existence such a regulatory mechanism, and to elucidate the physiological significance of the phosphorylation of tubulin by the tyrosine protein kinases.

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