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# $Ca^{2+}$/Calmodulin-Dependent Microtubule-Associated Protein 2 Kinase: Broad Substrate Specificity and Multifunctional Potential in Diverse Tissues†

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ABSTRACT: In previous studies, we described a soluble $Ca^{2+}$/calmodulin-dependent protein kinase which is the major $Ca^{2+}$/calmodulin-dependent microtubule-associated protein 2 (MAP-2) kinase in rat brain [Schulman, H. (1984) *J. Cell Biol. 99*, 11-19; Kuret, J. A., & Schulman, H. (1984) *Biochemistry 23*, 5495-5504]. We now demonstrate that this protein kinase has broad substrate specificity. Consistent with a multifunctional role in cellular physiology, we show that in vitro the enzyme can phosphorylate numerous substrates of both neuronal and nonneuronal origin including vimentin, ribosomal protein S6, synapsin I, glycogen synthase, and myosin light chains. We have used MAP-2 to purify the enzyme from rat lung and show that the brain and lung kinases have nearly indistinguishable physical and biochemical properties. A $Ca^{2+}$/calmodulin-dependent protein kinase was also detected in rat heart, rat spleen, and in the ring ganglia of the marine mollusk *Aplysia californica*. Partially purified MAP-2 kinase from each of these three sources displayed endogenous phosphorylation of a 54 000-dalton protein. Phosphopeptide analysis reveals a striking homology between this phosphoprotein and the 53 000-dalton autophosphorylated subunit of the major rat brain $Ca^{2+}$/calmodulin-dependent protein kinase. The enzymes phosphorylated MAP-2, synapsin I, and vimentin at peptides that are identical with those phosphorylated by the rat brain kinase. This enzyme may be a multifunctional $Ca^{2+}$/calmodulin-dependent protein kinase with a widespread distribution in nature which mediates some of the effects of $Ca^{2+}$ on microtubules, intermediate filaments, and other cellular constituents in brain and other tissues.

Although the molecular mechanisms underlying $Ca^{2+}$ action in most tissues remain elusive, a number of recent findings suggest some unifying principles. The discovery of calmodulin as a calcium-binding protein and as an activator of a cyclic nucleotide phosphodiesterase was followed by the recognition that a number of other proteins and enzymes were regulated by this protein (Cheung, 1980; Klee et al., 1980; Means et al., 1982). The finding that calmodulin activated a membrane-bound protein kinase(s) in brain and other mammalian tissues (Schulman & Greengard, 1978a,b) suggested that, by analogy with the elegant regulation of cellular processes by adenosine cyclic 3',5'-phosphate (cAMP)[1] via the cAMP-dependent

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protein kinase, some of the actions of $Ca^{2+}$ might also be coordinated by a "general" protein kinase, an enzyme with a broad, yet selective, substrate specificity. The existence of such a general $Ca^{2+}$-dependent kinase was an intriguing possibility (Greengard, 1978; Schulman, 1982, 1984a; Schulman & Greengard, 1978b; Walaas & Nairn, 1984). Two identified $Ca^{2+}$/calmodulin-dependent protein kinases, myosin light chain kinase and phosphorylase kinase, although certainly involved in mediating specific effects of $Ca^{2+}$, have a substrate specificity that is too limited to serve such a multifunctional role (Schulman, 1982). Appreciation of the difficulties inherent in studying the membrane-bound phosphorylation system led several investigators to search for a homologous enzyme in cytosol. In our studies on phosphorylation of the microtubule-associated protein 2 (MAP-2) in rat brain cytosol, we identified and purified a protein kinase responsible for $Ca^{2+}$/calmodulin-dependent phosphorylation of MAP-2 in brain (Schulman, 1984b,c; Schulman et al., 1983). Several findings led us to examine the possibility that the neuronal $Ca^{2+}$/calmodulin-dependent MAP-2 kinase might serve a more general role in mediating $Ca^{2+}$ action in brain and other tissues. These findings include the following: (a) examination of this activity in cytosol and in the purified state indicated that although MAP-2 was the predominant substrate, numerous other proteins were also phosphorylated (Kuret & Schulman, 1984; O'Callaghan et al., 1980; Schulman, 1984b: Walaas et al., 1983; (b) identification of apparently similar neuronal $Ca^{2+}$/calmodulin-dependent protein kinases which can phosphorylate MAP-2 although they were purified by using several different protein substrates (Bennett et al., 1983; Fukunaga et al., 1982; Goldenring et al., 1983; Kennedy et al., 1983; Nose & Schulman, 1982; Yamauchi & Fujisawa, 1983); (c) realization that the glycogen synthase kinase, a $Ca^{2+}$/calmodulin-dependent protein kinase from liver and skeletal muscle, had a broader substrate specificity than first realized (McGuiness et al., 1983; Schworer & Soderling, 1983) and had structural similarities to one of the neuronal enzymes (McGuiness et al., 1983); and (d) numerous reports of $Ca^{2+}$/calmodulin-dependent protein kinase activity in cytosolic extracts from various tissues (Freedman & Jamieson, 1982; Migliaccio et al., 1982; Palfrey, 1983; Schubart & Field, 1984; Spruill et al., 1983). These findings presented the possibility that such functions as the response of protein synthesis to secretagogues in exocrine pancreas, intermediate filament protein function in both testes and pancreatic islets, and estrogen receptor in uterus may be regulated by $Ca^{2+}$ via a $Ca^{2+}$/calmodulin-dependent protein kinase.

We report here that the neuronal $Ca^{2+}$/calmodulin-dependent MAP-2 kinase has a relatively broad, yet selective, substrate specificity. We show that MAP-2 kinase activity exists in cytosol from rat heart, rat lung, rat spleen, and ring ganglia of the marine mollusk *Aplysia californica*. The enzyme was purified from rat lung and compared to the brain enzyme by structural and functional criteria.

## Experimental Procedures

### *Materials*

Molecular weight markers aldolase, catalase, and thyroglobulin and the Sepharose 4B and 6B resins were purchased from Pharmacia Fine Chemicals. *Staphylococcus aureus* V8 protease was obtained from Miles Laboratories, Inc. DEAE-cellulose and phosphocellulose (P11) were purchased from Whatman Chemical Separation Inc. $NaDodSO_4$ electrophoresis reagents (other than acrylamide) were from Bio-Rad Laboratories. [$\gamma$-$^{32}$P]ATP (2000 Ci/mmol) was purchased from Amersham Corp., and all other reagents were obtained from Sigma Chemical Co. MAP-2, $\tau$, and tubulin were prepared from bovine brain as described (Schulman, 1984c). MAP-1 was prepared from bovine microtubules by the method of Vallee & Davis (1983), and rat brain fodrin was prepared essentially as described by Levine & Willard (1981). Calmodulin was prepared as described (Schulman & Greengard, 1978b).

Proteins used for analysis of substrate specificity were generously provided by the following individuals: Glycogen synthase from rabbit skeletal muscle was kindly provided by P. J. Roach, Indiana University. Synapsin I from bovine brain was the gift of Dr. T. Ueda, The University of Michigan. Ribosomes (40 S) were prepared from reticulocyte lysates and kindly given by Dr. J. Traugh, University of California, Riverside. Bovine lens vimentin was the gift of Drs. J. Nelson and E. Lazarides of the California Institute of Technology. Estrogen receptor purified from calf uterus was the gift of Dr. G. L. Green, The University of Chicago. Myosin light chains from turkey gizzard smooth muscle were a gift from Dr. R. Adelstein, National Institutes of Health, and light chains from rabbit skeletal muscle and bovine cardiac muscle were a gift of Dr. J. T. Stull, The University of Texas, and of A. Edelman and E. Krebs, University of Washington. Neurofilament proteins from rabbit spinal cord were a gift of M. Willard, Washington University. Chicken gizzard filamin and vinculin were the gift of Drs. D. K. Werth and Ira Pastan, National Institutes of Health.

Sepharose 6B gel filtration columns were calibrated by using porcine thyroglobulin ($M_r$ 669000, Stokes radius 85.0 Å), bovine catalase ($M_r$ 232000, Stokes radius 52.2 Å), and aldolase ($M_r$ 158000, Stokes radius 48.1 Å) as standards. The void volume ($V_0$) was estimated by elution volumes ($V_e$) by the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where $V_t$ = the total column volume. Molecular weights on $NaDodSO_4$ gels were calibrated by using phosphorylase *b* ($M_r$ 94000), bovine serum albumin ($M_r$ 67000), ovalbumin ($M_r$ 45000), trypsinogen ($M_r$ 25000), $\beta$-lactoglobin ($M_r$ 17500), and lysozyme ($M_r$ 14300) as standards. Protein was visualized by Coomassie Brilliant Blue R-250. Autoradiography was performed with Kodak BB-1 film (Eastman Kodak Co.) with or without a Coronex Lightning Plus intensifying screen (DuPont Instruments). Protein was routinely determined by the method of Bradford (1976) as modified by Spector (1978) using bovine serum albumin as the protein standard.

### *Methods*

*Substrate Specificity.* $Ca^{2+}$/calmodulin-dependent protein kinase activity toward putative substrates was determined by using a $NaDodSO_4$–polyacrylamide gel electrophoresis assay. The assay (final volume 100 $\mu$L) contained 50 mM Pipes (at the indicated pH), 15 mM $MgCl_2$, 0.2 mM EGTA (minus calcium), 0.2 mM EGTA + 0.5 mM $CaCl_2$ + 20 $\mu$g/mL calmodulin (plus calcium), 4.9 ng of $Ca^{2+}$/calmodulin kinase, protein substrate as indicated, and 20 $\mu$M [$\gamma$-$^{32}$P]ATP (0.5–2.5 Ci/mmol). Incubations were started by addition of [$\gamma$-$^{32}$P]-ATP and conducted at 30 °C for 3 min in duplicate, unless otherwise specified. Reactions were terminated by addition of 50 $\mu$L of a "$NaDodSO_4$ stop solution", heated for 2 min at 100 °C, and analyzed by $NaDodSO_4$–polyacrylamide gel

[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; MAP-2, microtubule-associated protein 2; $Cl_3CCOOH$, trichloroacetic acid; GABA, 4-aminobutyric acid.

electrophoresis (7, 9, or 12% acrylamide as appropriate) and autoradiography as described (Ueda & Greengard, 1977). Substrate proteins labeled with $^{32}P$ were localized by autoradiography of the dried gels. Phosphorylation was quantified by excising the $^{32}P$-labeled bands from the gel and by measuring Cerenkov radiation in a Packard TriCarb liquid scintillation spectrometer. Phosphorylation of casein was assayed by a $Cl_3CCOOH$ precipitation method as described (Kuret & Schulman, 1984).

*Purification of MAP-2 Kinase from Nonneuronal Tissues*: *(A) Purification of Rat Lung Enzyme.* $Ca^{2+}$/calmodulin-dependent MAP-2 kinase was purified from 12 g of rat lung essentially as described for purification of the rat brain enzyme (Schulman, 1984b), except for the following modifications. The cytosolic extract, prepared as described above, was made 10% (w/v) in glycerol, and the enzyme was maintained in glycerol throughout the purification. In addition, the order of the phosphocellulose and DEAE-cellulose steps was reversed, and the hydroxylapatite step was eliminated.

*(B) Partial Purification of Kinase from Rat Heart, Rat Spleen, and Aplysia Ganglia.* Cytosolic extracts were prepared from 1 g of rat heart and spleen as described above. Cytosolic extracts were prepared from ring ganglia of 40 *Aplysia* snails by collecting ganglia in artificial seawater at 4 °C over a 2-h period and homogenizing the rinsed ganglia using a Virtis Hi-Speed homogenizer at 50 000 rpm for 30 s followed by centrifugation at 150000*g* for 45 min. Cytosolic extract from each tissue was loaded onto a phosphocellulose column (1.4 × 5.5 cm) equilibrated with 25 mM Pipes (pH 7.0), 0.1 M NaCl, 2 mM EGTA, and 200 μM $PhCH_2SO_2F$ in 10% glycerol. The column was rinsed with 2 bed volumes of this buffer, and the enzyme was eluted by a 100-mL gradient of NaCl (0.1–1.0 M) in buffer B. MAP-2 kinase activity was eluted from the phosphocellulose column between 0.25 and 0.4 M NaCl. Pooled fractions were made 1 mM free $Ca^{2+}$, just before application on the calmodulin–Sepharose 4B affinity column (3 mL), and purified as previously described (Schulman, 1984c).

## Results

*Substrate Specificity of Rat Brain $Ca^{2+}$/Calmodulin-Dependent MAP-2 Kinase.* In previous studies, we identified and purified a $Ca^{2+}$/calmodulin-dependent protein kianse which is the major $Ca^{2+}$/calmodulin MAP-2 kinase in rat brain (Schulman, 1984b,c). The ability of this enzyme to phosphorylate a large number of endogenous substrates in rat brain cytosol indicated to us that this enzyme might have a broad substrate specificity. Reports in the literature showing $Ca^{2+}$/calmodulin-dependent phosphorylation of the intermediate filament protein vimentin (Spruill et al., 1983), of glycogen synthase (Ahmed et al., 1982; Payne et al., 1983; Woodgett et al., 1983), of ribosomal protein S6 (Freedman & Jamieson, 1982; Gorelick et al., 1983), and of the estrogen receptor (Migliaccio et al., 1982) with the use of extracts or purified enzyme from Sertoli cells, muscle and liver, exocrine pancreas, and uterus, respectively, suggested a number of possible substrates to test with the neuronal MAP-2 kinase. The result of the analysis of substrate specificity of the rat brain $Ca^{2+}$/calmodulin-dependent MAP-2 kinase is shown in Table I. Indeed, the neuronal enzyme has a relatively broad substrate specificity. Although purified by its ability to phosphorylate MAP-2, it phosphorylates a number of other substrates quite well. The velocity given in the table is not a $V_{max}$ because several of the substrates could not be tested at concentrations higher than their respective $K_m$'s. In addition, as noted previously (Kuret & Schulman, 1984), the pH profile of this enzyme displays a marked dependence on substrate. A consensus value of pH 7.6 was used where feasible in order to compare substrates under similar conditions. Thus, no attempt is made to provide an absolute rank order of preferred substrates. The analysis does show, however, that substrates such as glycogen synthase, synapsin I (Ueda & Greengard, 1977), myosin light chains from smooth muscle, vimentin, and ribosomal protein S6 are good substrates of the neuronal MAP-2 kinase and thus have the potential of being physiological substrates of a similar $Ca^{2+}$/calmodulin-dependent protein kinase in their respective tissues. A number of cytoskeletal proteins, including vinculin, filamin, fodrin, neurofilament proteins (subunits of 200 000, 145,000, and 68,000 daltons) and MAP-1 (subunits of 350 000, 30 000, and 28 000 daltons), were found to incorporate negligible amounts of phosphate despite the fact that most of them have been shown to be excellent substrates for other protein kinases. When tested at 0.2 mg/mL, each incorporated phosphate at less than 0.5 nmol $mg^{-1}$ $min^{-1}$. Several other proteins tested were heavily phosphorylated (greater than 0.6 mol of phosphate/mol of subunit) although their rate of phosphorylation was not determined under conditions used for the substrates in Table I. These proteins include the estrogen receptor from calf uterus, the microtubule-associated proteins collectively called τ, and a polypeptide modulator of the GABA receptor called GABA–modulin (Wise et al., 1983). Thus, the neuronal MAP-2 kinase has a broad, yet selective, substrate specificity.

Table I: Substrate Specificity of Purified $Ca^{2+}$/Calmodulin Kinase

| substrate | concn (mg/mL) | purity (%) | velocity (nmol $mg^{-1}$ $min^{-1}$)[a] |
|---|---|---|---|
| glycogen synthase | 0.02 | 100 | 336 |
| synapsin I | 0.08 | 50 | 280 |
| MAP-2 | 0.2 | 46 | 268 |
| vimentin | 0.2 | 100 | 136 |
| myosin light chains, smooth muscle | 0.2 | 50 | 82 |
| ribosomal protein S6 | 1.0 | 2 | 80 |
| casein | 0.2 | 100 | 24 |
| tubulin | 0.5 | >95 | 23 |
| myosin light chains | | | |
| skeletal muscle | 0.2 | >90 | 11 |
| cardiac muscle | 0.2 | >90 | 5 |

[a] Reaction rates were determined at pH 7.6 for all substrates except for the light chains which were assayed at pH 7.2.

*Purification of MAP-2 Kinase from Rat Lung.* The ability of the neuronal MAP-2 kinase to phosphorylate a number of substrates from nonneuronal tissues, and the reports of $Ca^{2+}$/calmodulin-dependent protein kinase activity in nonneuronal and nonmammalian tissues, suggested that an enzyme similar to the MAP-2 kinase purified from rat brain may exist outside of the mammalian central nervous system. We chose rat lung as a representative nonneuronal tissue and examined its MAP-2 kinase in greater detail. The rat lung $Ca^{2+}$/calmodulin-dependent MAP-2 kinase activity was purified essentially as described earlier for the rat brain enzyme (Experimental Procedures). Throughout the purification, the rat lung enzyme displayed the same chromatographic properties as the rat brain enzyme, suggesting that they have similar biophysical properties. The analysis of the penultimate purification step, gel filtration, is shown in Figure 1. Column fractions were assayed for MAP-2 kinase activity and quantified as described under Experimental Procedures. The MAP-2 kinase activity was eluted as a symmetrical peak centering at 102 mL, corresponding to a holoenzyme with a molecular weight of 610 000 based on globular protein

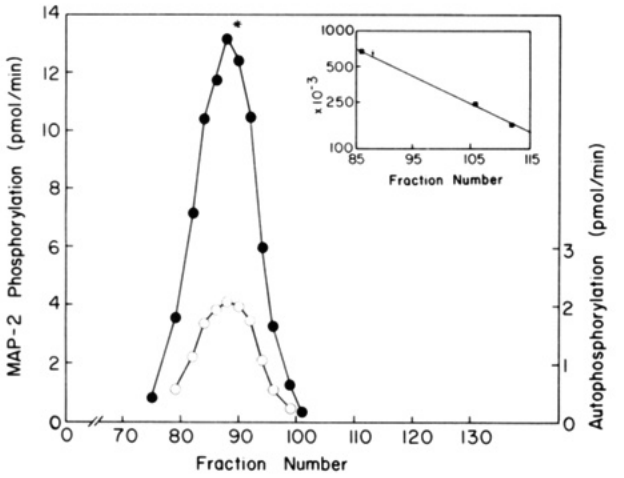


FIGURE 1: Elution profile of autophosphorylated kinase and of MAP-2 kinase activity chromatographed on Sepharose 6B. The $Ca^{2+}$/calmodulin-dependent MAP-2 kinase eluting from the phosphocellulose column was concentrated by ammonium sulfate, dialyzed, and chromatographed on a column of Sepharose 6B (1.6 × 82 cm). Aliquots were assayed for MAP-2 kinase activity (●) and separately for the phosphorylation of the putative autophosphorylated subunit at 53 000 daltons (○) in the presence of calcium plus calmodulin as described under Experimental Procedures. The asterisk shows the position of rat brain kinase chromatographed separately on the same column. The inset shows elution of the lung MAP-2 kinase relative to the protein standards (■) (1) porcine thyroglobulin ($M_r$ 669 000), (2) bovine catalase ($M_r$ 232 000), and (3) aldolase ($M_r$ 158 000). The arrow indicates the position of the activity peak.

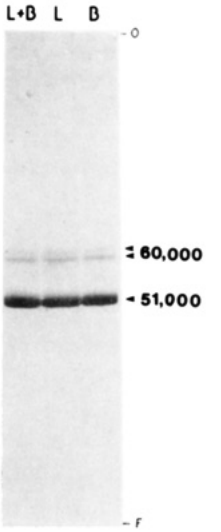


FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified $Ca^{2+}$/calmodulin-dependent protein kinase from rat lung and rat brain. An aliquot of the purified $Ca^{2+}$/calmodulin-dependent protein kinase from rat lung, rat brain, and a mixture of the two was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (9% acrylamide) as described under Experimental Procedures. The lanes contain 5 μg of lung kinase (L), 4 μg of brain kinase (B), and 2.5 μg of lung kinase plus 2 μg of brain kinase (L + B). Protein, stained with Coomasssie Brilliant Blue, is shown with molecular weights of kinase subunits indicated. The top of the gel is indicated by O and the position of the dye front (bromphenol blue) with F.

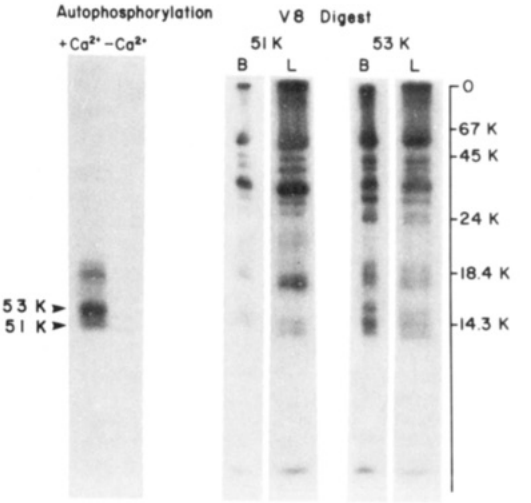


FIGURE 3: $Ca^{2+}$/calmodulin-dependent autophosphorylation of rat lung kinase. (a) Purified rat lung $Ca^{2+}$/calmodulin-dependent protein kinase (100 ng) was incubated for 5 min in the absence of substrate and in the presence ($+Ca^{2+}$) or absence ($-Ca^{2+}$) of calcium plus calmodulin to assay for autophosphorylation (Experimental Procedures). Autoradiograph of reaction mixture resolved on 9% $NaDodSO_4$ gels is shown. (b) Putative autophosphorylated subunits at 51 000 daltons (L, 51K) and at 53 000 daltons (L, 53K) from the gel above were subjected to partial proteolysis in 15% $NaDodSO_4$ gels using *S. aureus* V8 protease as described under Experimental Procedures and compared to the corresponding brain kinase autophosphorylated subunits (B, 51K and B, 53K) prepared similarly.

standards. This kinase is therefore rather similar to the rat brain enzyme which displayed a molecular weight by gel filtration of 580 000. Analysis for endogenous phosphorylation in the presence of $Ca^{2+}$ and calmodulin and in the absence of exogenous substrate revealed the putative autophosphorylated subunits at a similar molecular mass (53 000 daltons) to that of the rat brain enzyme. Quantification of the autophosphorylated subunits is shown in Figure 1. These polypeptides comigrate on Sepharose 6B at a constant ratio to the MAP-2 kinase activity, as seen earlier with the brain enzyme. On the last step of the purification, the enzyme bound to calmodulin–Sepharose and could not be eluted with 0.5 M NaCl if calcium was also present. The enzyme was eluted from the calmodulin–Sepharose column with EGTA, consistent with its being a calmodulin-binding protein. The $Ca^{2+}$/calmodulin-dependent MAP-2 kinase from rat lung was purified to near-homogeneity. Comparison of the subunit composition of the rat lung enzyme with the rat brain enzyme is shown in Figure 2. The rat lung enzyme contains a major band at 51 000 daltons and minor bands of 60 000 daltons (a doublet), 71 000 daltons, and several at approximately 300 000 daltons. The 51 000-dalton polypeptide and the 60 000-dalton doublet appear indistinguishable from the major subunits of the rat brain kinase (Figure 2). Subsequent analysis indicates, in fact, that these polypeptides are subunits of the lung kinase. It is not known whether the other polypeptides, particularly at 71 000 daltons, are subunits of the lung eznyme or contaminants of the preparation. The purified enzyme has a Stokes radius of 81.3 ± 2.7 Å determined by gel filtration and a sedimentation coefficient of 15.1 ± 0.65 S determined by sedimentation velocity on 5–20% sucrose gradients. The native molecule weight was calculated to be 519 000 by the method of Siegel & Monty (1966).

*Comparison of Brain and Lung Enzymes: Phosphopeptide Analysis.* The purified kinase was assayed for endogenous phosphorylation by incubation under standard conditions in the absence of MAP-2. In the presence of $Ca^{2+}$ plus calmodulin, the enzyme displayed marked incorporation of $^{32}P$ into polypeptides of 51 000 and 53 000 daltons and into a broad band at 64 000 daltons (Figure 3a). This is quite similar to what is seen with the brain enzyme, where these phosphoproteins have been demonstrated to result from autophosphorylation of the 51 000- and 60 000-dalton subunits of the kinase (Kuret & Schulman, 1984). In the case of the rat brain enzyme, phosphorylation of these subunits increases their apparent molecular weight on $NaDodSO_4$–polyacrylamide gel

Table II: Comparison of Substrate Specificity of Purified Kinases from Brain and Lung

| substrate | concn (mg/mL) | brain/lung ratio[a] |
|---|---|---|
| ribosomal protein S6 | 1.0 | 1.25 |
| phosvitin | 0.5 | 1.15 |
| vimentin | 0.2 | 1.02 |
| MAP-2 | 0.2 | (1.0) |
| myosin light chains, smooth muscle | 0.2 | 0.93 |
| synapsin I | 0.08 | 0.85 |
| glycogen synthase | 0.1 | 0.70 |

[a] Ratios of reaction rates are normalized to give MAP-2 a ratio of 1.0.

electrophoresis to 53 000 and 64 000, respectively. Phosphorylation of the lung enzyme was absolutely dependent on the presence of both $Ca^{2+}$ and calmodulin.

The phosphorylated 51 000-dalton subunit, migrating as phosphoproteins at 51 000 and 53 000 daltons, was compared with the corresponding subunit of the brain enzyme by partial proteolysis using *Staphylococcus aureus* V8 protease (Cleveland et al., 1977; Schulman, 1984c). This comparison (Figure 3b), using purified enzymes, demonstrates a striking similarity in phosphopeptide pattern between the brain and lung enzymes. Almost every phosphopeptide seen in the lung enzyme is also seen in the brain enzyme. This suggests a rather strong homology between the 51 000-dalton subunit of the lung and brain enzymes.

*Comparison of Lung and Brain Enzymes: Substrate Specificity.* Functional comparison of the $Ca^{2+}$/calmodulin-dependent protein kinases from rat brain and lung was performed by examining their substrate specificity. The initial velocity of phosphorylation of a number of identified brain kinase substrates was determined and the ratio presented in Table II. MAP-2 and the other proteins found to be good substrates of the brain enzyme are, in fact, good substrates of the lung enzyme.

Since many of these substrates have multiple sites of phosphorylation, the possibility remained that, although the rates of phosphorylation by the two enzymes were similar, the enzymes were phosphorylating different sites on the substrate proteins. The site specificity of the brain and lung enzymes was compared. Two of the best substrates, MAP-2 and synapsin I, were phosphorylated to similar extents by the brain and lung enzymes. The phosphorylated polypeptides were then subjected to partial proteolysis. As shown in Figure 4, the same phosphopeptide pattern is generated by using either kinase. A 34 000-dalton fragment was the major phosphopeptide derived from synapsin I. The rat lung kinase therefore phosphorylates the same portion of synapsin I that is phosphorylated by the major soluble rat brain enzyme and a portion distinct from that phosphorylated by the cAMP-dependent protein kinase and by another $Ca^{2+}$/calmodulin-dependent protein kinase (Kennedy & Greengard, 1981; Nairn & Greengard, 1983). Similarly, phosphopeptides ranging from 9000 to 23 000 daltons are characteristic of MAP-2 phosphorylated by the neuronal MAP-2 kinase (Schulman, 1984b) and are found without exception in MAP-2 phosphorylated by the lung kinase. Partial proteolysis of vimentin phosphorylated by either of the two enzymes also generates identical phosphopeptides (data not shown).

*MAP-2 Kinase in Heart, Spleen, and Aplysia Ganglia.* We undertook the purification of a $Ca^{2+}$/calmodulin-dependent MAP-2 kinase from several rat tissues to determine the tissue distribution of the kinase. The ability of the purified rat brain enzyme to autophosphorylate its 51 000-dalton subunit, producing a phosphoprotein migrating at 53 000 daltons (Bennett et al., 1983; Kuret & Schulman, 1984), offered a convenient way to specifically label and compare kinases from the various tissues without the need for complete purification. Examination of endogenous phosphorylation in cytosol from rat heart and spleen and from *Aplysia* ganglia revealed $Ca^{2+}$-dependent phosphorylation of a polypeptide of approximately 54 000 daltons. MAP-2 kinase activity was detected in each of the cytosolic extracts (data not shown). A two-step procedure was therefore developed for partial purification of the MAP-2 kinase activity by chromatography on phosphocellulose and calmodulin–Sepharose in order to enrich for the calmodulin-binding kinase and thus facilitate the comparisons (Experimental Procedures). The MAP-2 kinase activity was eluted from the phosphocellulose at the same NaCl concentration needed for elution of the rat brain enzyme and was eluted from the affinity column only in the presence of EGTA. Examination of endogenous phosphorylation of the partially purified preparations obtained after the calmodulin–Sepharose step reveals the presence of a $Ca^{2+}$/calmodulin-dependent protein kinase activity with a major substrate at 54 000 daltons (Figure 5). Other rat tissues, including lung, skeletal muscle, testes, and exocrine pancreas, showed similar patterns of endogenous phosphorylation (data not shown).

We next tested the possibility that the 54 000-dalton phosphoprotein in the partially purified enzyme from rat heart, spleen, and *Aplysia* ganglia was related to the autophosphorylated rat brain kinase by comparing the sites of phosphorylation in the protein and the substrate specificity of the kinases. The 54 000-dalton phosphopeptide from each of the three tissues was excised from the gel and subjected to partial proteolysis using *S. aureus* V8 protease as previously described (Cleveland et al., 1977; Schulman, 1984c). The results shown in Figure 6 demonstrate a marked similarity between the phosphopeptide pattern of the putative kinase band from rat heart and spleen and *Aplysia* ganglia. All three resemble the purified rat brain MAP-2 kinase used as a

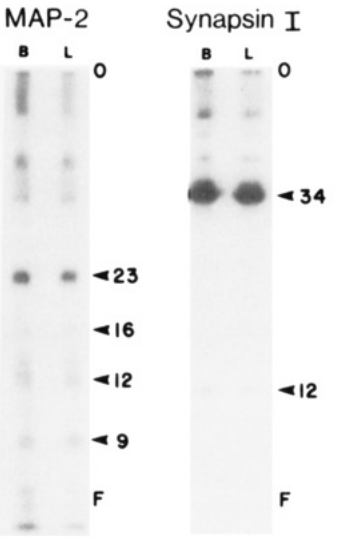


FIGURE 4: Phosphopeptide specificity of rat and lung kinases. A preparation of bovine brain MAP-2 (4 μg) and synapsin I (1 μg) was phosphorylated by the purified $Ca^{2+}$/calmodulin-dependent protein kinases from brain (40 ng) and lung (50 ng) and analyzed by Na-$DodSO_4$–polyacrylamide gel electrophoresis. The $^{32}P$-labeled MAP-2 and synapsin Ia were excised from the gels, and partial proteolysis was performed as described. Similar results were obtained with partial proteolysis of either synapsin Ia or synapsin Ib. Autoradiographs corresponding to MAP-2 and synapsin I phosphorylated by the brain (B) and lung (L) kinases are shown above. Molecular weights ($\times 10^{-3}$) of relevant phosphopeptides are indicated.

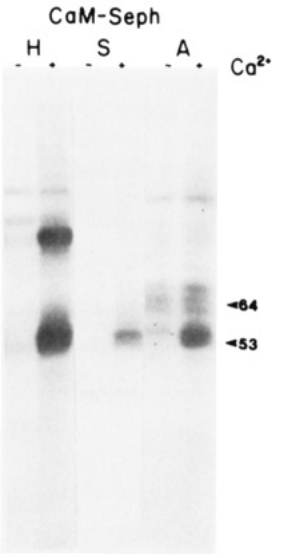


FIGURE 5: Autoradiograph of $Ca^{2+}$/calmodulin-dependent phosphorylation in rat heart, rat spleen, and *Aplysia* ganglia. Cytosolic extracts and partially purified $Ca^{2+}$/calmodulin-dependent protein kinase were prepared as described under Experimental Procedures. Aliquots of the calmodulin–Sepharose pool (CaM–Seph) (heart, 4 μg; spleen, 4 μg; *Aplysia* ganglia, 1 μg) were assayed for endogenous phosphorylation in the absence (−) and presence (+) of calcium plus calmodulin and analyzed on 9% $NaDodSO_4$ gels. Samples from heart (H) and spleen (S) were run on a separate gel than those from *Aplysia* ganglia (A). Arrowheads indicate positions of the 53 000- and 64 000-dalton autophosphorylated subunits of the brain $Ca^{2+}$/calmodulin-dependent protein kinase.

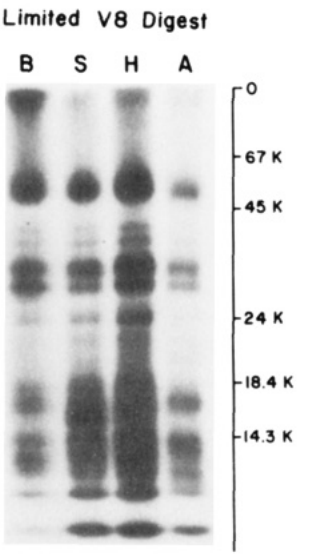


FIGURE 6: Partial proteolysis of a common $^{32}P$-labeled polypeptide from rat brain, heart and spleen and from *Aplysia* ganglia. Endogenous calcium-dependent phosphorylation with two-step-purified $Ca^{2+}$/calmodulin kinase from rat heart and spleen and *Aplysia* ring ganglia and with purified MAP-2 kinase from rat brain was carried out as described in the legend to Figure 5. The putative autophosphorylated band was analyzed by partial proteolysis using 2 μg of *S. aureus* V8 protease per lane in 12.5% $NaDodSO_4$–polyacrylamide gels. The four lanes are of the 54 000-dalton phosphoprotein from rat spleen (S), rat heart (H), and *Aplysia* ganglia (A) and of the 53 000-dalton rat brain phosphoprotein (B) used for comparison.

standard. The major phosphopeptides derived from the brain enzyme are also present in the phosphoprotein from the other tissues. A similar analysis for the heart and spleen enzyme using chymotrypsin also demonstrated extensive homology with the brain enzyme (data not shown). Like the brain enzyme, the other MAP-2 kinases had an absolute dependence for both $Ca^{2+}$ and calmodulin. In addition, these enzymes phosphorylated MAP-2 at sites identical with those previously characterized as the preferred sites of phosphorylation by the purified rat brain $Ca^{2+}$/calmodulin-dependent protein kinase and distinct from the sites phosphorylated by the cAMP-dependent protein kinase (data not shown).

## Discussion

We report here that the $Ca^{2+}$/calmodulin-dependent MAP-2 kinase, previously described in rat brain (Schulman, 1984b,c; Walaas et al., 1983), exists in several nonneuronal rat tissues and in the one nonmammalian tissue tested. This study was prompted, in part, by reports of MAP-2 in nonneuronal tissues (Cleveland et al., 1979), which led us to suspect that a $Ca^{2+}$-dependent MAP-2 kinase similar to the brain enzyme might also be present in other tissues to regulate the nonneuronal MAP-2. We demonstrate that such nonneuronal tissues indeed contain a $Ca^{2+}$-dependent MAP-2 kinase with considerable homology to the neuronal kinase. The MAP-2 kinase purified from rat lung shows structural, physical, and functional similarities to the brain kinase. The 51 000-dalton polypeptide and 60 000-dalton doublet, previously identified as subunits of the neuronal MAP-2 kinase holoenzyme (Schulman, 1984c), are present in the highly purified preparations from lung. In addition to these, the lung enzyme contains a polypeptide with a molecular mass of 71 000 daltons which may be either a contaminant of the preparation or a subunit not represented in the neuronal enzyme. The two kinases have similar molecular weights, are absolutely dependent on $Ca^{2+}$ plus calmodulin for activity, undergo an apparent autophosphorylation, and have similar phosphopeptide patterns generated by proteolysis of the autophosphorylated bands. In this respect, these two enzymes also share considerable homology with the partially purified preparation of MAP-2 kinase from heart and spleen.

The neuronal MAP-2 kinase has a broad, yet selective, substrate specificity. Its activity in vivo may therefore not be limited to regulation of MAP-2 function, although it is interesting that several other cytoskeletal proteins, including τ and vimentin, are good in vitro substrates. This MAP-2 kinase constitutes approximately 0.3% of the cytosolic proteins in rat brain (Bennett et al., 1983; Schulman, 1984c). The concentration of this enzyme in nonneuronal cell cytosol is difficult to estimate because of the relatively high basal level of phosphorylation contributed by other protein kinases. However, on the basis of the activity of the purified or partially purified preparations described here, a measurement that is highly affected by differences in yields, we estimate that nonneuronal rat tissues contain at most 10% of the brain level of this kinase. This disparity may be related to the high concentration of MAP-2 and other cytoskeletal proteins in brains relative to other tissues.

Some distinct differences between the brain and other MAP-2 kinases are apparent. Whereas the lung enzyme shows subunits of both 51 000 and 60 000 daltons, the heart, spleen, and *Aplysia* ganglia enzymes do not show a prominent band corresponding to the 60 000-dalton subunit. In addition, the smaller subunit, which migrates as a 53 000-dalton phosphoprotein in brain, appears to be a 54 000-dalton phosphoprotein in heart, spleen, and *Aplysia* ganglia. Since in the latter tissues the kinase was only partially purified and detected by its apparent autophosphorylation, it is not known whether the differences result from different extents of phosphorylation, and thus of migration on $NaDodSO_4$ gels, or from true differences in their size. The autophosphorylation has permitted the detection of low levels of kinase and provides a convenient labeling of kinase subunits for structural comparisons. We

have demonstrated previously that the phosphopeptide patterns of the 51 000- and 60 000- dalton subunits generated by either *S. aureus* V8 protease or chymotrypsin are distinct but similar (Kuret & Schulman, 1984). The 53 000- or 54 000-dalton subunit in the various tissues tested here is quite homologous to the brain enzyme, although some differences can be seen that may represent minor structural variations expected of isozymes. In the case of the heart, spleen, and *Aplysia* ganglia, for which the kinases were only partially purified, some of the phosphopeptides may result from phosphoprotein contaminants migrating with the 54 000-dalton autophosphorylated subunit. Some differences in substrate specificity are seen, although the values are remarkably similar when contrasted to the substrate specificity of several other protein kinases (Beavo & Mumby, 1982; Carlson et al., 1979; Glass & Krebs, 1980; Hathaway & Traugh, 1982). Despite these slight differences, the enzymes appear to be functionally similar as evidenced by their ability to phosphorylate the same fragments of MAP-2, synapsin I, and vimentin.

A comparison of several documented $Ca^{2+}$/calmodulin-dependent protein kinases in rat brain cytosol with the rat brain MAP-2 kinase described here suggests that they are likely to be the same enzyme [see Discussion in Schulman (1984c)]. One other $Ca^{2+}$/calmodulin-dependent protein kinase, designated as type I (Nairn & Greengard, 1983), does not phosphorylate MAP-2 while phosphorylating synapsin I and is clearly distinct from the other kinases. Recent studies demonstrate that the glycogen synthase from rabbit skeletal muscle, although lacking a 51 000-dalton subunit, shares considerable homology in its 58 000-dalton subunit with the corresponding subunit of the neuronal synapsin I kinase (McGuiness et al., 1983). Similarly, homogenates of *Aplysia* ganglia have recently been shown to phosphorylate a 51 000-dalton protein which has a similar phosphopeptide pattern to the neuronal synapsin I kinase (DeRiemer et al., 1984).

$Ca^{2+}$/calmodulin-dependent protein kinase activity, distinct from myosin light chain kinase and phosphorylase kinase, has been described in various tissues. In the absence of more detailed biochemical characterization of many of these enzymes, it is impossible to assess their relationship to the neuronal and nonneuronal MAP-2 kinase described here. It is perhaps not coincidental, however, that each of the proteins found to be substrates of a $Ca^{2+}$ kinase in a nonneuronal tissue, e.g., vimentin in testes, ribosomal protein S6 in exocrine pancreas, estrogen receptor in uterus, and glycogen synthase in liver and muscle, is also a good substrate for the brain MAP-2 kinase. We have demonstrated that rat heart, spleen, and lung cytosol contain a MAP-2 kianse similar to the $Ca^{2+}$/calmodulin-dependent kinas from brain. It is therefore likely that the kinases responsible for the phosphorylation of the various substrates described above are closely related to the MAP-2 kinase described here.

Each of the substrates of the $Ca^{2+}$/calmodulin-dependent protein kinase has been identified as a substrate of other protein kinases. For example, vimentin is phosphorylated subsequent to elevation of cAMP, presumably by the cAMP-dependent protein kinase (Groppi & Browning, 1980; O'Connor et al., 1981), as well as during mitosis by a cAMP-independent protein kinase (Evans, 1984). Ribosomal protein S6 phosphorylation is stimulated by extracellular signals such as insulin and epidermal growth factor (EGF) (Perisic & Traugh, 1983; Thomas et al., 1982) and in vitro by a variety of protein kinases activated by cAMP, insulin, and $Ca^{2+}$ plus diglyceride (Donahue & Masaracchia, 1984; LePeuch et al., 1983; Perisic & Traugh, 1983; Traugh et al., 1973). It is not surprising that proteins involved in critical control of functions such as shape, motility, or protein synthesis would be subject to complex regulation. $Ca^{2+}$-dependent phosphorylation provides an additional level of regulation. The various protein kinases may phosphorylate the same or different sites on these substrate proteins and thus provide a mechanism for integrating the effects of the extracellular signals.

The neuronal and nonneuronal $Ca^{2+}$/calmodulin kinases may all be isozymes or tissue-specific forms of the same protein kinase. They have the in vitro characteristics of "general" protein kinases. By analogy to the cAMP-dependent protein kinases, these enzymes would respond to changes in intracellular free $Ca^{2+}$ concentration and mediate some of the actions of $Ca^{2+}$ in their respective tissues. They would function independently or in concert with the $Ca^{2+}$/diglyceride-dependent protein kinase (Nishizuka, 1984; Schulman, 1984a) to mediate specific effects of a variety of hormones, neurotransmitters, growth factors, and other extracellular signals that elevate diglyceride and/or $Ca^{2+}$.

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**Registry No.** MAP-2 kinase, 97350-82-8; glycogen synthase, 9014-56-6.

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