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A MAP-2-Stimulated Protein Kinase Activity Associated with Neurofilaments†

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ABSTRACT: Preparations of mammalian neurofilaments purified from brain consist primarily of four proteins: a triplet of molecular weights 210000, 160000, and 69000, and tubulin. Incubation of bovine brain neurofilaments in the presence of adenosine 5′-[γ-^{32}P]triphosphate ([γ-^{32}P]ATP) brought about phosphorylation of each of these four proteins, indicating that a protein kinase was associated with the neurofilaments. The activity was not removed by centrifugal washing of the neurofilaments in a number of different buffers. The pattern, rate, and extent of phosphorylation were found to be unaffected by adenosine cyclic 3′,5′-monophosphate (cAMP), by preincubation with adenosine 5′-diphosphate, or by addition of a protein kinase inhibitor. The protein kinase is thus cAMP independent. When the high molecular weight microtubule-associated protein MAP-2 was added to neurofilaments and the mixture was incubated with [γ-^{32}P]ATP, the MAP-2 became extensively phosphorylated. In addition, the inclusion of MAP-2 brought about an increase in the extent of phosphorylation of the neurofilament proteins. These results show that neurofilaments possess a firmly associated protein kinase activity and that this activity is stimulated by the presence of MAP-2. Microtubule preparations made by cycles of assembly and disassembly can contain both protein kinase activity and small amounts of contaminating neurofilaments. Two distinct protein kinases were resolved from such microtubule protein by gel filtration on Bio-Gel A-150m. The cAMP-independent protein kinase activity was found in the void volume fractions, together with the contaminating neurofilaments. A cAMP-dependent protein kinase activity was found in the included peak, together with the major proteins of microtubules. The neurofilament-associated protein kinase is thus distinct from, and separable from, the microtubule-associated protein kinase.

Neurofilaments are found in axons in close proximity to microtubules [for reviews, see Dustin (1978) and Lasek & Hoffman, (1976)]. They contain a triplet of proteins of molecular weights approximately 69000, 160000, and 210000 (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976; Schlaepfer, 1978; Micko & Schlaepfer, 1978). In addition, tubulin accompanies them in axonal transport (Lasek & Hoffman, 1976) and through isolation under a variety of conditions (Runge et al., 1979a,b, 1981). Hence, tubulin may be a part of neurofilaments. The functions of neurofilaments are not known, although they are clearly transported in the slow component of axonal transport (Hoffman & Lasek, 1975; Lasek & Hoffman, 1976).

The ability to prepare neurofilaments in tens of milligrams (Runge et al., 1981) has allowed investigation of their biochemical properties. A phosphodiesterase has been found (Runge et al., 1979b) to be firmly associated with neurofilaments purified by the methods employed in the present study. The protein kinase activity reported below was found in a search for other neurofilament-associated enzymic activities, undertaken with the eventual intention of elucidating the functions of these organelles.

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Microtubule preparations contain cAMP[1]-dependent protein kinase activities (Soifer et al., 1972, 1975; Soifer, 1973; Sloboda et al., 1975; Sheterline, 1977) that catalyze phosphorylation of exogenous test substrates as well as endogenous proteins. The apparent preferred substrate of microtubule-associated protein kinase, both in vitro and in vivo, is the microtubule-associated protein MAP-2[2] (Sloboda et al., 1975). Because neurofilaments mingle with microtubule components in the brain homogenate, because tubulin and perhaps traces of other microtubular proteins contaminate neurofilament preparations (Runge et al., 1981), and because neurofilaments can contaminate microtubule preparations (Berkowitz et al., 1977; Keats & Hall, 1975), it is necessary to establish carefully whether a neurofilament-associated activity is unique to that structure or is related to the microtubule-associated enzyme. Separation of neurofilaments from the other components of cycled microtubule preparations has been accomplished by gel filtration on Bio-Gel A-150m (Berkowitz et al., 1977), which has an exclusion limit of $\sim 1.50 \times 10^8$ molecular weight. This separation was employed here to determine if a protein kinase activity is associated with neurofilaments when they are separated from the other proteins of a crude microtubule preparation.

A preliminary report of this work has appeared (Runge et al., 1979c). A protein kinase associated with guinea pig neurofilaments has been mentioned in abstract (Shecket & Lasek, 1979).

Materials and Methods

Pipes,[1] EGTA, DTE, GTP, ATP, and cAMP were obtained from Sigma Biochemicals, St. Louis, MO. Bio-Gels A-150m and A-1.5m were obtained from Bio-Rad Laboratories, Richmond, CA. Urea ("ultrapure") was from Mann Research Laboratories, New York.

All experiments involving neurofilament protein were performed in 0.1 M Pipes (pH 6.9), 2 mM EGTA, 1 mM $MgSO_4$, and 2 mM DTE (referred to as PM buffer) at 4 °C unless otherwise specified.

Isolation of Neurofilaments from Bovine Brain. Isolation and purification of neurofilaments was carried out by differential centrifugation and gel filtration, exactly as described by Runge et al. (1981). Before use, aliquots of the frozen droplets were quickly thawed and centrifuged (1000g × 10 min) to remove small amounts of irreversibly aggregated material.

Protein Kinase Activity Assay. Both cAMP-dependent and cAMP-independent protein kinase activities were measured by the method of Corbin & Reimann (1974). Standard reaction mixtures contained neurofilaments, or microtubule protein, at 0.25–1.5 mg/mL, in either 0.05 M Pipes (pH 6.9), 1 mM EGTA, 0.5 mM $MgSO_4$, and 1 mM DTE or 25 mM Tris-HCl (pH 7.0) and 0.5 mM $MgCl_2$. All reaction mixtures contained 0.4 mM [γ-^{32}P]ATP (20–50 cpm/pmol). For determination of the pH optimum, mixtures were made 25 mM in Tris-HCl, at values of the pH between 5.0 and 9.0. For investigation of the influence on protein kinase activity of various cations, neurofilament protein in 25 mM Tris-HCl, pH 7.0, was made 2.5 mM in either $MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnCl_2$, $FeCl_2$, or $CaCl_2$. Experiments were performed in the presence and absence of 2 μM cAMP, or of 2 units of protein kinase inhibitor (Walsh et al., 1971), to assay for the presence of cAMP-dependent protein kinase activity. Incubations were performed at 30 °C, and reactions were started by the addition of enzyme (either neurofilament or microtubule protein). Twenty-microliter samples were removed at 5, 15, 30, 45, and 60 min, or at 10 and 20 min, and were applied to filter paper disks which were subsequently treated to remove unincorporated phosphate. Radioactivity of the disks was measured by liquid scintillation counting. Aliquots of the assay mixture were also counted to determine the specific radioactivity of the [γ-^{32}P]ATP.

Microtubule Preparation. Cycled microtubule protein[3] was prepared by the assembly–disassembly method of Shelanski et al. (1973) as modified by Berkowitz et al. (1977). Twice-cycled microtubule preparations that contained substantial amounts of neurofilaments were prepared by the use of short clarification centrifugations (30 min × 95500g) at 4 °C. Neurofilaments were obtained from these preparations by gel filtration on Bio-Gel A-150m as described (Runge et al., 1979a). When a sample was assayed for protein kinase activity, aliquots were saved for gel electrophoresis. Protein concentrations were determined by the method of Bradford (1976) and standardized with tubulin as described by Detrich & Williams (1978).

Preparation of MAP-2. MAP-2 was isolated by the method of Kim et al. (1979). Twice-cycled microtubule protein was heated to 100 °C for 5 min and cooled on ice for 15 min. The precipitated protein was removed by centrifugation at 40000g for 20 min at 4 °C. The supernatant, which contained the MAP-2, was concentrated by precipitation in 50% ammonium sulfate. The precipitated protein was resuspended, dialyzed into PM buffer plus 0.75 M NaCl, and chromatographed on a column of Bio-Gel A-1.5m (2.5 × 60 cm). Void volume fractions were collected, assayed for purity by gel electrophoresis, and pooled.

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) on 8% cylindrical or slab gels. Gels were stained with Coomassie Brilliant Blue R. Gels with ^{32}P-labeled protein were first scanned for absorbance at 595 nm and then sliced into ~40 3-mm slices which were counted for radioactivity.

$NaDodSO_4$–urea–polyacrylamide gradient slab gel electrophoresis was carried out as described by Kim et al. (1979). This method is similar to that described by Laemmli (1970) except that the gels contain a 4–16% acrylamide gradient, a 1–8 M urea gradient, and no $NaDodSO_4$ in either the stacking or the separating gel. Gels with ^{32}P-labeled protein were stained in Coomassie Brilliant Blue R, destained, and wrapped with cellophane for autoradiography. The cellophane-wrapped gels were placed directly on a sheet of Kodak Xs5 X-ray film for 2–7 days for exposure.

Results

Presence of Protein Kinase Activity. Figure 1 shows the incorporation of phosphate into the proteins of a neurofilament

[1] Abbreviations used: ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-monophosphate; DTE, dithioerythritol; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid; GTP, guanosine 5′-triphosphate; HMW, high molecular weight; MAP's, microtubule-associated proteins; PC, phosphocellulose; Pipes, piperazine-N,N'-bis(2-ethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; ADP, adenosine 5′-diphosphate.

[2] MAP-2 refers to one of the high molecular weight microtubule-associated proteins (Sloboda et al., 1975; Kim et al., 1979).

[3] Microtubule protein refers to tubulin plus all associated proteins (MAP's) that copurify with tubulin through cycles of assembly and disassembly in vitro. Tubulin refers specifically to the α–β dimer of the major microtubule protein.

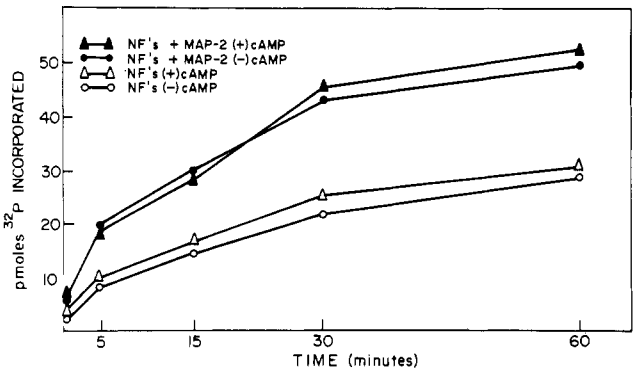

FIGURE 1: Protein kinase activity in bovine brain neurofilament preparations. Phosphorylation of endogenous protein was measured in the presence and absence of 2 μM cAMP and with or without 0.20 mg/mL added MAP-2. The assay mixtures contained 0.05 M Pipes (pH 6.9), 1 mM EGTA, 0.5 mM $MgSO_4$, 1 mM DTE, and 1.0 mg/mL neurofilament protein. Samples (20 μL) were applied to filter paper and counted as described under Materials and Methods.

Table I: Effect of Protein Kinase Inhibitor (PK_i) and Preincubation with ADP on Phosphorylation of Endogenous Protein and MAP-2[a]

sample	activity	activity (+PK_i)	activity (+PK_i, +cAMP)	activity (preincubation)	activity (preincubation, +cAMP)
NF's	51 ± 4.9	45 ± 4.0	47 ± 6.5	44 ± 2.7	49 ± 4.1
NF's + MAP-2	85 ± 10.5	88 ± 8.6	91 ± 11.2	86 ± 7.9	90 ± 9.9

[a] The assay mixtures consisted of 1.25 mg/mL neurofilaments (NF's) in PM buffer to which had been added 0.4 mM [γ-^{32}P]ATP (20–50 cpm/mol). cAMP (2.0 μM) and MAP-2 (0.21 mg/mL) were present where indicated. In some experiments, neurofilament protein was dialyzed into PM buffer containing 2 mM ADP (two changes of 1 L each at 4 °C). These solutions were warmed to 30 °C for 30 min and were then dialyzed back into PM buffer lacking ADP. The dialyzed samples were then assayed as described above.

preparation incubated with [γ-^{32}P]ATP under four different conditions. Phosphorylation of protein clearly occurred, and its rate was not significantly affected by the addition of cAMP. Thus, a protein kinase is clearly present in these neurofilament preparations, and it is not cAMP dependent. Figure 1 also shows that addition of MAP-2 to the reaction mixture increases the amount of phosphorylation ~2-fold at each of the times measured. This change must result either from a simple increase in the number of sites available for phosphorylation or from a stimulation of the protein kinase, or from both causes. The role of MAP-2 in the protein kinase mediated reaction will be described below.

The protein kinase activity remains bound to the neurofilaments throughout the course of their preparation. For investigation of whether high concentrations of salt would remove the enzyme, ~2 mg of neurofilaments was carried through several cycles of centrifugal pelleting (106000g × 1 h at 4 °C) and resuspension in 1 mL of a PM buffer to which 0.264 M NaCl had been added to yield a total ionic strength of 0.5 M. In the final pellet after three such washes, 53%; of the total protein and 79% of the protein kinase activity were recovered. Small amounts of activity (<5% of the total) were lost in two subsequent washes. It thus appears that the majority of the activity remains with the neurofilaments under conditions of high ionic strength, even though some other proteins dissociate from them.

Table I shows the effects of the Walsh protein kinase inhibitor, as well as the effects of preincubation in ADP, on the

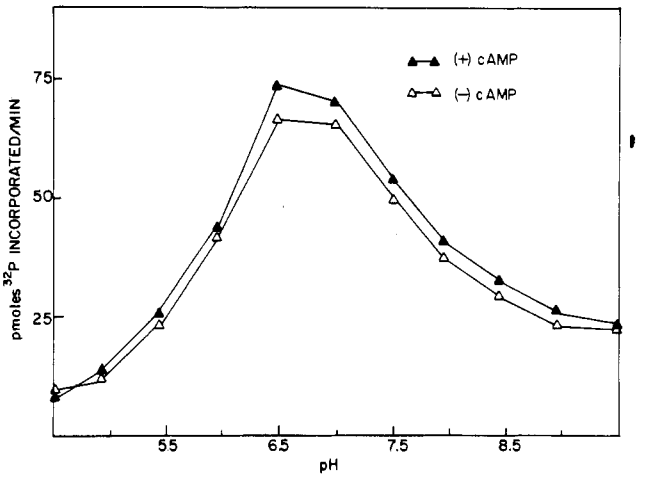

FIGURE 2: Phosphorylation of endogenous protein as a function of pH. The pH dependence of the neurofilament-associated protein kinase was determined in the presence and absence of 2 μM cAMP. Twenty-microliter samples of assay mixtures containing 25 mM Tris-HCl, pH 5.0–9.0, 0.5 mM $MgCl_2$, and 1.0 mg/mL neurofilament protein were applied to filter papers for counting.

Table II: Effect of Divalent Cations on Phosphorylation of Endogenous Protein[a]

ion	protein kinase activity (pmol of ^{32}P incorpd/min) −cAMP	protein kinase activity (pmol of ^{32}P incorpd/min) +cAMP
Mg^{2+}	47 ± 2.3	44 ± 4.1
Co^{2+}	32 ± 3.7	32 ± 6.0
Mn^{2+}	25 ± 1.7	28 ± 2.5
Fe^{2+}	20 ± 2.3	23 ± 1.7
Zn^{2+}	21 ± 3.8	21 ± 2.7
Ca^{2+}	12 ± 3.5	15 ± 4.2

[a] The divalent cations listed above were added, as their chlorides, to 2.5 mM in reaction mixtures containing 25 mM Tris-HCl, pH 7.0, 0.4 mM [γ-^{32}P]ATP (20–50 cpm/pmol), and 1.0 mg/mL neurofilament protein. The reaction mixtures were incubated for 10 min at 30 °C and then counted, as described under Materials and Methods. The assays were performed in duplicate with each of three different neurofilament preparations, and the numbers above represent the mean and range of the determinations. The indicated samples contained 2 μM cAMP.

Table III: Effect of Calmodulin on Neurofilament-Associated Protein Kinase[a]

sample	activity	activity (+Ca^{2+})	activity (+cAMP)	activity (+Ca^{2+}, +cAMP)
NF's	43 ± 4.7	35 ± 3.6	47 ± 3.6	36 ± 2.8
NF's + calmodulin	41 ± 6.1	32 ± 2.9	44 ± 4.2	38 ± 1.9
NF's + calmodulin + MAP-2	94 ± 12.6	75 ± 6.5	90 ± 9.1	76 ± 8.4

[a] The assay mixtures contained PM buffer, 0.4 mM [γ-^{32}P]ATP (20–50 cpm/pmol), and 2 μM cAMP, 50 μM Ca^{2+}, 0.25 mg/mL MAP-2, 25 μg/mL calmodulin, and 1.5 mg/mL neurofilament protein as indicated. Samples were incubated for 10 and 20 min at 30 °C, and aliquots were removed and counted as described under Materials and Methods. Assays were performed in duplicate with two different neurofilament preparations.

phosphorylation reaction. Neither addition significantly altered the protein kinase activity, either in the presence of in the absence of MAP-2, thus indicating that the neurofilament-associated enzyme is intrinsically cAMP independent.

Figure 2 shows that the protein kinase is active over a broad range of pH, with an optimum near pH 6.5. Activity of the enzyme in the presence of a number of divalent cations is

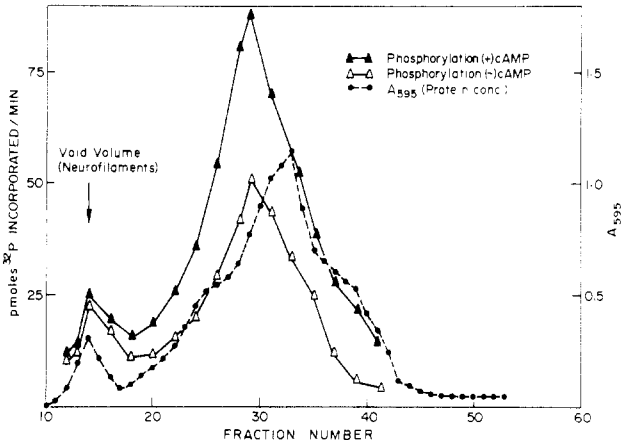

FIGURE 3: Chromatography of microtubule protein on Bio-Gel A-150m. Microtubule proteins were fractionated into a void volume peak (containing neurofilaments) and an included peak. Rings were localized on the leading edge of the included peak by electron microscopy. Five milliliters at 10 mg/mL microtubule protein was loaded onto a 2.5 × 30 cm column of Bio-Gel A-150m. Fractions of 1.5 mL were collected. Aliquots of fractions were assayed in 0.05 M Pipes (pH 6.9), 1 mM EGTA, 0.5 mM $MgSO_4$, and 1 mM DTE, in the presence and absence of 2 μM cAMP. In addition, aliquots of fractions 13–16 and 26–31 were assayed in the presence of 1 unit of protein kinase inhibitor. No difference in activity was observed in fractions 13–16. The protein kinase activity of fractions 26–31 was reduced to 6–10 pmol of ^{32}P incorporated/min. Similar results (see text) were observed when aliquots of the same fractions were dialyzed against PM buffer plus 2 mM ADP, warmed to 30 °C for 30 min, dialyzed back into PM buffer, and assayed.

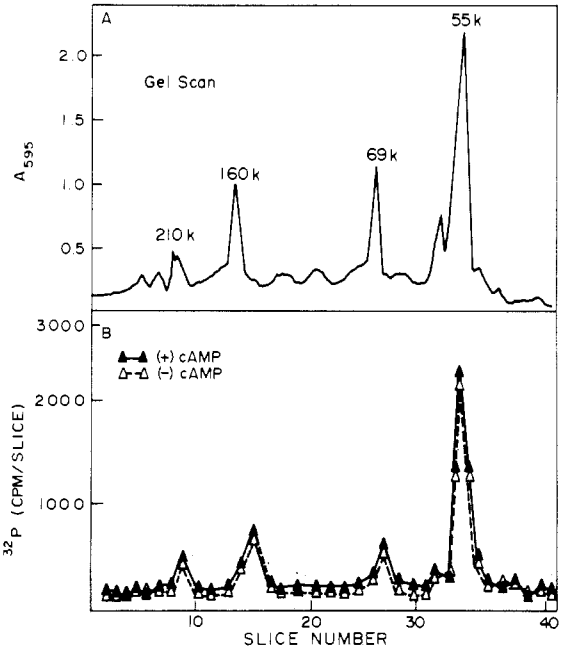

FIGURE 4: Phosphorylation of endogenous protein by neurofilament-associated protein kinase. An aliquot of neurofilaments was incubated with 0.4 mM [γ-^{32}P]ATP in the presence and absence of 2 μM cAMP. Samples contained 1.5 mg/mL neurofilament protein in PM buffer. Approximately 30 μg of protein was loaded onto each tube gel. Gels were stained with Coomassie Brilliant Blue R, destained, and scanned in a GCA/McPherson Model EU 701 spectrophotometer equipped with a linear transport. The gels were then sliced into 3-mm slices, placed in 5 mL of ACS scintillation fluid, and counted.

reported in Table II. Activity is highest in the presence of Mg^{2+} and is inhibited, comparatively, by Ca^{2+}.

Results of an investigation of the capacity of the protein kinase to be activated by Ca^{2+} via calmodulin are shown in Table III. No significant activation by calmodulin and Ca^{2+} is evident, either in the presence or in the absence of MAP-2. The inhibition by Ca^{2+} apparent in the data of Table II is seen here also, as is the lack of effect of cAMP, previously shown in Figure 1. The lack of an effect of calmodulin is not an artifact of the presence of Mg^{2+} in the buffer, since the affinity of calmodulin for Ca^{2+} is 1000-fold higher than its affinity for Mg^{2+}, while the concentration of Ca^{2+} is only 10-fold less than that of Mg^{2+} in the experiment.

Two Protein Kinases in Cycled Microtubule Preparations. For clarification of the relationship of the neurofilament-associated protein kinase to the cAMP-dependent protein kinase found in microtubules prepared by cycles of assembly and disassembly, a preparation of twice-cycled microtubule protein was fractionated by gel filtration on Bio-Gel A-150m. Protein kinase activity was assayed across the chromatogram, in the absence and in the presence of cAMP, with the results shown in Figure 3. Gel filtration divides the activity into two major peaks. The first peak, which coincides with that of the neurofilaments, was not stimulated significantly by cAMP. The second peak of activity coincides with the leading edge of the included protein peak, and it is activated ~2-fold by cAMP. The activity of the enzyme in tubes 26–31 was reduced ~5-fold from its maximum level to a level of 6–10 pmol/min, when 1.0 unit of Walsh protein kinase inhibitor was included in the cAMP-containing incubation mixture. No significant effect of inhibitor on the protein kinase in tubes 13–16 was observed. These results show that cycled microtubule preparations, which contain neurofilaments, contain both cAMP-dependent and cAMP-independent protein kinase activities. The two enzymes are distinct and separable.

Identity of Phosphorylated Proteins. For determination of which of the endogenous proteins are phosphorylated by the neurofilament-associated kinase, aliquots of neurofilaments were incubated at 30 °C for 60 min with 0.4 mM [γ-^{32}P]ATP were then subjected to $NaDodSO_4$ electrophoresis. Figure 4A shows the absorbance profile of a Coomassie Blue stained gel. The triplet of neurofilament proteins, with molecular weights of 210 000, 160 000, and 69 000, together with tubulin, accounted for ~85% of the total protein. (The two minor polypeptides with molecular weights of >210 000 comigrate with MAP-2 from microtubule preparations.) Figure 4B shows the distribution of ^{32}P in two samples, one incubated in the presence of cAMP and one in its absence. The majority of label was incorporated into proteins whose positions corresponded to tubulin and the neurofilament triplet. Little or no effect of cAMP on the amount of phosphorylation is apparent. Although the absorbance of Coomassie Blue stain is not strictly proportional to the amount of protein in the gel, a rough estimate of the amount of ^{32}P incorporation per unit mass of protein can be made by comparison of parts A and B of Figure 4. No major differences in extent of phosphorylation are visible between the four proteins.

Figure 5A shows a scan of a stained $NaDodSO_4$ electrophoresis gel prepared from a sample of neurofilaments incubated with [γ-^{32}P]ATP in the presence of added MAP-2. The same amount of protein as in Figure 4 was electrophoresed here. The added MAP-2 is visible as a doublet of molecular weight >210 000. Figure 5B shows the distribution of ^{32}P among the proteins of two identically incubated aliquots, one containing cAMP and one lacking it. MAP-2 is seen to be extensively phosphorylated: although it represented only 14% of the total protein present, it received >50% of the incorporated phosphate. There was, again, no detectable effect of cAMP. As a control, MAP-2 preparations were assayed for protein kinase activity. None was expected because of the heat treatment involved in purification of MAP-2, and none was found.

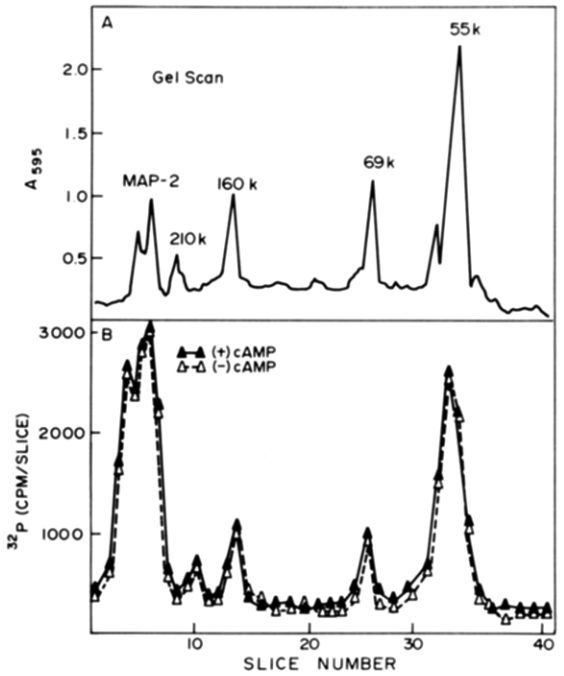

FIGURE 5: Phosphorylation of endogenous proteins and MAP-2 by neurofilament-associated protein kinase. Neurofilament preparations were incubated with 0.4 mM [γ-^{32}P]ATP in the presence and absence of 2 μM cAMP. Samples contained 1.5 mg/mL neurofilament protein and 0.25 mg/mL MAP-2 in PM buffer. Approximately 30 μg of protein was loaded onto each tube gel. Gels were stained, destained, scanned, and counted as described in Figure 4.

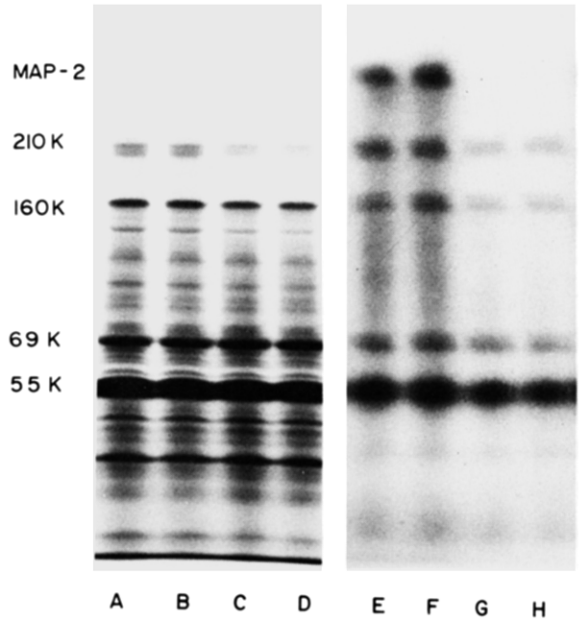

FIGURE 6: Phosphorylation of endogenous proteins and MAP-2 by neurofilament-associated protein kinase. Samples were incubated and prepared as described in Figure 5 with the exception that the concentration of neurofilament protein was 2.3 mg/mL and that of MAP-2 was 0.19 mg/mL in this experiment. Approximately 50 μg of protein was loaded into each slot and electrophoresed on a polyacrylamide–urea gradient slab gel (see Materials and Methods). (A–D) Coomassie-stained samples: (A) neurofilaments plus MAP-2 plus cAMP; (B) neurofilaments plus MAP-2; (C) neurofilaments plus cAMP; (D) neurofilaments. (E–H) Autoradiography of the samples in (A–D). The gel was stained and destained with Coomassie Brilliant Blue R, wrapped in cellophane, and autoradiographed as described under Materials and Methods.

Stimulation of Neurofilament-Associated Protein Kinase by MAP-2. The increase in protein kinase activity that was observed to occur in the presence of MAP-2 (Figure 1) was investigated further. Figure 6 shows $NaDodSO_4$ gel electrophoresis of neurofilaments incubated with ATP in the presence and absence of cAMP and in the presence and absence of added MAP-2. Both the stained gel (A–D) and its autoradiogram (E–H) are shown. Comparison of the amounts of protein with the extents of phosphorylation shows clearly that the addition of a small amount of MAP-2 to the phosphorylation mixture has sharply increased the extent of phosphorylation of tubulin and the neurofilament proteins. The same effect can be seen less directly by comparing Figure 4B with Figure 5B. The extensive phosphorylation of MAP-2 itself can be seen by comparison of parts A and B of Figure 6 with parts E and F. These results show, first, that MAP-2 is a comparatively good substrate for the neurofilament-associated protein kinase and, second, that MAP-2 activates the protein kinase. Neither of these two effects is altered by cAMP.

Discussion

The data show that a protein kinase activity is associated with neurofilaments prepared from bovine brain. Whether this enzyme is an intrinsic component of the neurofilaments is not certain, although all the results obtained thus far are consistent with the hypothesis that it is. The enzymic activity is not dissociated from neurofilaments by the centrifugation and gel filtration procedures used to isolate them. It also is not removed from neurofilaments by the exposures to varied temperatures (0–34 °C) and the incubations in glycerol-containing buffers that are employed in the preparation of microtubules. It is not removed by repeated washes at 0.5 M ionic strength. Proof that the enzyme adheres to neurofilaments in vivo will have to await studies by intracellular labeling techniques, such as immunofluorescence methods. However, the firm association in vitro reported here makes it seem likely that the protein kinase belongs to the filaments and makes it seem unlikely that it adheres accidentally at some time after disruption of the cells.

The separation by gel filtration (Figure 3) of the neurofilament-associated protein kinase from the microtubule-associated protein kinase demonstrates that they are different enzymes. The neurofilament-associated activity is shown in this and the other experiments (Figures 1, 2, 4, and 5 and Table I) to be cAMP independent. The lack of effect of the Walsh inhibitor shows that it is not simply a cAMP-dependent enzyme that has lost its regulatory subunits during preparation but that it belongs to the class of truly cAMP-independent protein kinases. It is likely that the microtubule-associated protein kinase activity is the same as that reported by Sloboda et al. (1975) and by Rappaport et al. (1976) and that it also corresponds to the cAMP-dependent fraction of the kinase activity noted by Sheterline (1977). The activity described by these workers catalyzes negligible amounts of phosphorylation of tubulin: Sloboda et al. (1975) estimated that 650 times as many moles of ^{32}P were incorporated per mole of MAP-2 as per mole of tubulin during brief phosphorylation in vitro. The neurofilament-associated kinase, in contrast, shows only an approximately 5–10-fold preference for MAP-2 and thus catalyzes substantial phosphorylation of tubulin.

The finding that MAP-2 stimulates the activity of the neurofilament-associated protein kinase is of particular interest. It suggests that a component of microtubules may modulate an enzymic function of neurofilaments. The close apposition of the two structures in the cell (Peters et al., 1970; Lasek & Hoffman, 1976; Dustin, 1978) and the geometrical position of MAP-2 as an external projection of the microtubule (Dentler et al., 1975; Murphy & Borisy, 1975; Sloboda et al., 1976; Herzog & Weber, 1978; Kim et al., 1979) render such

an interaction possible. The present data do not show, however, whether MAP-2 will stimulate the protein kinase when the MAP-2 has been incoporated into the microtubular wall. Neither do they show whether the phosphorylation of MAP-2 is related in any way to its capacity to stimulate protein kinase.

The pattern of phosphorylation of proteins in vivo by the neurofilament-associated kinase is not known and will be difficult to unravel because of the presence of the microtubule-associated enzyme. Both activities catalyze phosphorylation of MAP-2, although they may act at different loci on the MAP-2 molecule. The phosphorylation of tubulin in vivo may well be catalyzed in part by the neurofilament-associated enzyme. It appears that tubulin can be phosphorylated in vivo (Reddington et al., 1976; Forgue & Dahl, 1979; Eipper, 1974), although the extent of net phosphorylation appears to be small (Sloboda et al., 1975; Rappaport et al., 1976). The preferential phosphorylation in vivo of the β chain of tubulin noted by Forgue and Dahl and by Eipper is not visible in our results, however, owing partly, perhaps, to incomplete resolution of the chains of tubulin (see Figure 6).

The neurofilament-associated protein kinase reported here is similar to the guinea pig enzyme reported in abstract by Schecket & Lasek (1979) in being apparently filament-bound and in its ability to phosphorylate the proteins of the neurofilament triplet. It also resembles, in its Mg^{2+} and Ca^{2+} dependence, in its cAMP independence, and in its capacity to catalyze phosphorylation of neurofilaments, the protein kinase activity recently reported to be present in squid axoplasm (Pant et al., 1979). Whether protein kinases will turn out to be general features of all neurofilaments, and whether they are intrinsic to neurofilaments, remains to be determined.

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