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Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I–actin interactions

(synaptic transmission/synaptic plasticity/synaptic vesicle proteins/signal transduction/synaptogenesis)

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ABSTRACT **The ability of neurotrophins to modulate the survival and differentiation of neuronal populations involves the Trk/MAP (mitogen-activated protein kinase) kinase signaling pathway. More recently, neurotrophins have also been shown to regulate synaptic transmission. The synapsins are a family of neuron-specific phosphoproteins that play a role in regulation of neurotransmitter release, in axonal elongation, and in formation and maintenance of synaptic contacts. We report here that synapsin I is a downstream effector for the neurotrophin/Trk/MAP kinase cascade. Using purified components, we show that MAP kinase stoichiometrically phosphorylated synapsin I at three sites (Ser-62, Ser-67, and Ser-549). Phosphorylation of these sites was detected in rat brain homogenates, in cultured cerebrocortical neurons, and in isolated presynaptic terminals. Brain-derived neurotrophic factor and nerve growth factor upregulated phosphorylation of synapsin I at MAP kinase-dependent sites in intact cerebrocortical neurons and PC12 cells, respectively, while KCl-induced depolarization of cultured neurons decreased the phosphorylation state at these sites. MAP kinase-dependent phosphorylation of synapsin I significantly reduced its ability to promote G-actin polymerization and to bundle actin filaments. The results suggest that MAP kinase-dependent phosphorylation of synapsin I may contribute to the modulation of synaptic plasticity by neurotrophins and by other signaling pathways that converge at the level of MAP kinase activation.**

Neurotrophic factors and neurotransmitters have been shown to initiate signaling cascades that result in the activation of a family of proline-directed protein kinases known as mitogen-activated protein kinases (MAP kinases), or extracellular signal-regulated kinases (ERKs) (1–6). Given the diversity of cellular effects that are mediated by the activation of MAP kinases, it seems likely that the specificity imparted to neuronal MAP kinase signaling is attributable, at least in part, to the presence of neuron-specific substrates for this enzyme. Synapsin I, a member of a family of neuron-specific phosphoproteins that are localized on small synaptic vesicles (7, 8), is a physiological substrate for cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases) (9). In adult synapses, a variety of evidence is consistent with the hypothesis that synapsin I tethers synaptic vesicles to the actin cytoskeleton in a phosphorylation-dependent manner and through this mechanism regulates the proportion of vesicles in the nerve terminal that are available for release (9–12). Synapsin–actin interactions are also believed to contribute to the effects of the synapsins on synapse formation (13–17). In the present study, we have investigated the phosphorylation of synapsin I by MAP kinase *in vitro* and in intact neuronal preparations and have examined the consequence of this phosphorylation on the ability of synapsin I to interact with actin.

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MATERIALS AND METHODS

***In Vitro* Phosphorylation of Synapsin I by MAP Kinase and Identification of MAP Kinase-Dependent Phosphorylation Sites.** Synapsin I was purified from rat and bovine brain as described (18). MAP kinase, $p44^{mpk}$, and the cyclin-dependent protein kinase (cdk1)–cyclin A complex were purified from sea star oocytes and assayed as described (19, 20) by using 50 μM [γ-^{32}P]ATP (DuPont/NEN) and 5 μM synapsin I. For stoichiometric phosphorylation, reactions were carried out for 2 hr with 7 μM synapsin I in the absence (MOCK-P) or presence of the indicated protein kinase. Samples were subjected to SDS/PAGE, followed by staining with Coomassie blue. Incorporation of ^{32}P was quantitated by using a PhosphorImager (Molecular Dynamics). Two-dimensional phosphopeptide map analysis and phosphoamino acid analysis (21) and in-gel MAP kinase assays (22) were performed as described. For sequence determination, rat synapsin I (340 μg) was stoichiometrically phosphorylated with $p44^{mpk}$ in the presence of trace amounts of [γ-^{32}P]ATP and digested for 36 hr at 37°C in a buffer containing 100 mM Tris (pH 8), 10% (vol/vol) CH_3CN, 1% hydrogenated Triton X-100, and 17 μg each of trypsin and chymotrypsin; 1 M urea was added after 18 hr. ^{32}P-labeled phosphopeptides were purified in a two-step procedure by reversed-phase HPLC using a C_{18} column (0.46 × 15 cm, Vydac, Hesperia, CA). Two major ^{32}P-labeled peaks were isolated by linear gradient elution in the first chromatographic step [buffer 1: 10 mM potassium phosphate (pH 2.2) with an increasing concentration of 40% CH_3CN/20% isopropanol]. Peaks 1 and 2 were further processed with a different buffer system (buffer 2: 0.1% trifluoroacetic acid with increasing concentrations of 70% CH_3CN). Peak 1 was resolved into two ^{32}P-labeled phosphopeptides (peaks 1A and 1B), which appeared to be pure on the basis of absorbance profiles at 214 nm. Peak 2 was eluted as a single ^{32}P-labeled peak in the second step. Each phosphopeptide was derivatized with ethanethiol (23) prior to automated Edman degradation. The sequence obtained for peak 1A corresponded to residues 533–554 of rat synapsin I, with phosphoserine at residue 549; peak 1B corresponded to residues 54–76, with phosphoserine at residues 62 and 67; peak 2 corresponded to residues 54–73, with phosphoserine at residues 62 and 67.

Abbreviations: MAP kinase, mitogen-activated protein kinase; BDNF, brain-derived neurotrophic factor; cdk, cyclin-dependent protein kinase; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; PKA, cAMP-dependent protein kinase; NGF, nerve growth factor.

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Production of Phosphorylation State-Specific Antibodies and Immunoblot Analysis. Phosphorylation state-specific antibodies for the MAP kinase-dependent phosphorylation sites in synapsin I were produced as described (24, 25). A peptide corresponding to residues 58–72 of synapsin I was chemically phosphorylated at residues Ser-62 and Ser-67 (referred to as P-sites 4 and 5, respectively) and was employed to generate rabbit polyclonal antibodies that specifically detected phosphorylation at these sites (P-site 4/5 Ab; G-526). A phosphopeptide corresponding to residues 545–555 of rat synapsin I was synthesized with phosphoserine at residue 549 (referred to as P-site 6) and used to generate antibodies that specifically detected phosphorylation at site 6 (P-site 6 Ab; G-555). Antisera were screened by immunoblot analysis with purified samples (100 ng) of dephospho-synapsin I, synapsin I phosphorylated by MAP kinase, and synapsin I phosphorylated at sites 1, 2, and 3 by PKA plus CaM kinase II. Each antibody was specific for MAP kinase-phosphorylated synapsin I (Fig. 1*B*). The specificity of the P-site 4/5 Ab and P-site 6 Ab for their particular sites was confirmed by V8 protease digestion of MAP kinase-phosphorylated synapsin I, which generated an N-terminal fragment containing sites 4 and 5 and a C-terminal fragment containing site 6 (not shown). Immunoblot analysis was carried out as described (25) by using P-site 4/5 Ab and P-site 6 Ab (1:100 dilution), or anti-ERK1-antibody (1:500 dilution; K-23, Santa Cruz Biotechnology), followed by ^{125}I-labeled anti-rabbit IgG (Amersham). In Figs. 2–4, lanes containing purified bovine synapsin I phosphorylated by MAP kinase (100 ng) are indicated by "std." Lanes containing control samples [no treatment with brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF)] are indicated by "c." Quantification of immunoblots was accomplished with a PhosphorImager.

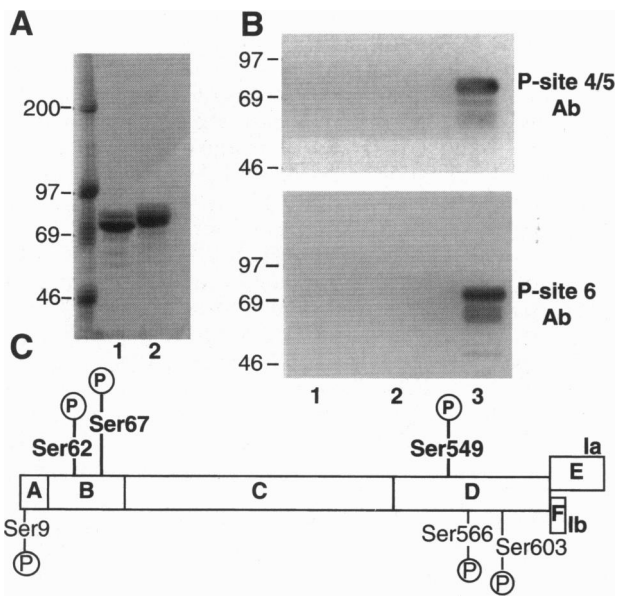

FIG. 1. MAP kinase phosphorylates Ser-62, Ser-67, and Ser-549 in rat synapsin I. (*A*) Migration of the distinct doublet of dephospho-synapsin Ia and Ib (lane 1) was retarded upon phosphorylation by p44^{mpk} (lane 2), resulting in broad bands of higher apparent molecular mass. Bands were visualized by Coomassie blue. (*B*) Samples (100 ng) of dephospho-synapsin I (lane 1), synapsin I phosphorylated by PKA plus CaM kinase II (lane 2), and synapsin I phosphorylated by MAP kinase (lane 3) were subjected to SDS/PAGE and immunoblot analysis using the P-site 4/5 Ab or P-site 6 Ab. (*C*) Relative location of six phosphorylation sites in synapsin I. Ser-9 (site 1) is phosphorylated by PKA and CaM kinases I and IV (26); Ser-566 (site 2) and Ser-603 (site 3) are phosphorylated by CaM kinases II and IV (26); and Ser-62 (site 4), Ser-67 (site 5), and Ser-549 (site 6) are phosphorylated by MAP kinase. Site 6 is also phosphorylated by cdk1 (27) and cdk5 (not shown).

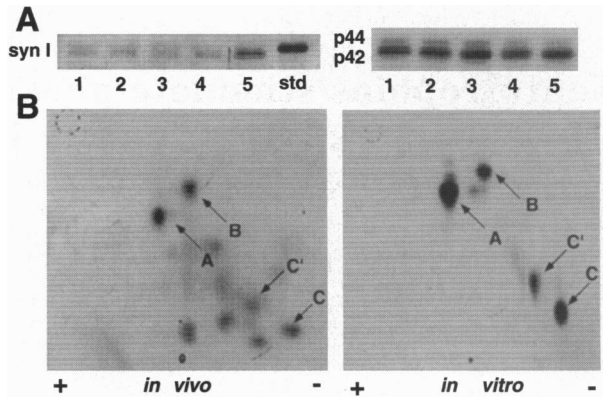

FIG. 2. (*A*) Immunoblot analysis of the distribution of MAP kinase phosphorylated synapsin I using P-site 4/5 Ab (*Left*) and MAP kinase isoforms, p44 and p42 (*Right*), in subcellular fractions of rat brain. SDS extracts (40 μg) of homogenate (lane 1), S1 (lane 2), S2 (lane 3), P2 (lane 4), and purified synaptosomes (lane 5) were subjected to SDS-PAGE. Bovine phosphosynapsin I standard (std; 100 ng/lane) migrates with a slightly higher apparent molecular mass than the rat isoform. (*B*) Two-dimensional phosphopeptide maps of ^{32}P-labeled synapsin I phosphorylated in purified synaptosomes (*Left*) and *in vitro* by MAP kinase (*Right*). Phosphopeptide A corresponded to HPLC peak 2 (residues 54–73); phosphopeptide B corresponded to HPLC peak 1B (residues 54–76); phosphopeptide C corresponded to HPLC peak 1A (residues 533–554); phosphopeptide C′ was a cyclized form of phosphopeptide C, with a pyroglutamyl residue at the N terminus.

Preparation and ^{32}P-Prelabeling of Synaptosomes. Synaptosomes from rat cerebral cortex were purified and prelabeled with ^{32}Pi (DuPont/NEN) at 1 mCi/ml (1 Ci = 37 GBq) as described (28, 29). ^{32}P-labeled synapsin I was immunoprecipitated and subjected to SDS/PAGE and two-dimensional phosphopeptide map analysis (21).

Cell Culture. Embryonic day 18 rat cerebrocortical tissue was used to prepare primary neuronal cultures as described (30). Cultures were maintained in serum-free medium for 5 days and then analyzed. PC12 cells were grown and maintained as described (31). Hu-recombinant BDNF was supplied by Regeneron, and NGF (2.5S) was purchased from GIBCO/BRL.

Actin Bundling and Polymerization Assays. Purification of actin, derivatization with *N*-(1-pyrenyl)iodoacetamide, and fluorescence measurements of polymerization were done as described (32, 33). Actin bundling was analyzed by light-scattering assays and electron microscopy as described (18).

RESULTS

Synapsin I Is Phosphorylated by MAP Kinase at Three Sites *in Vitro*. *In vitro*, synapsin I was found to be an excellent substrate for sea star MAP kinase, p44^{mpk}. Under initial rate conditions for synapsin I phosphorylation, nonlinear double reciprocal plots were obtained. However, an estimate of $K_{0.5}$ of synapsin I for MAP kinase was in the range of 20–50 μM, and the rate was comparable to that observed for myelin basic protein in assays run in parallel. The stoichiometry of phosphorylation reached a maximal level of 3 mol of phosphate per mol of synapsin I (data not shown). Phosphorylation of synapsin I by MAP kinase caused a reduction in electrophoretic mobility (Fig. 1*A*), which was not seen when synapsin I was phosphorylated at site 1 by PKA and/or at sites 2 and 3 by CaM kinase II or at site 6 only by cdk1. Phosphoamino acid analysis demonstrated that only seryl residues were phosphorylated (data not shown). Phosphopeptide map analysis of synapsin I phosphorylated by MAP kinase revealed a specific

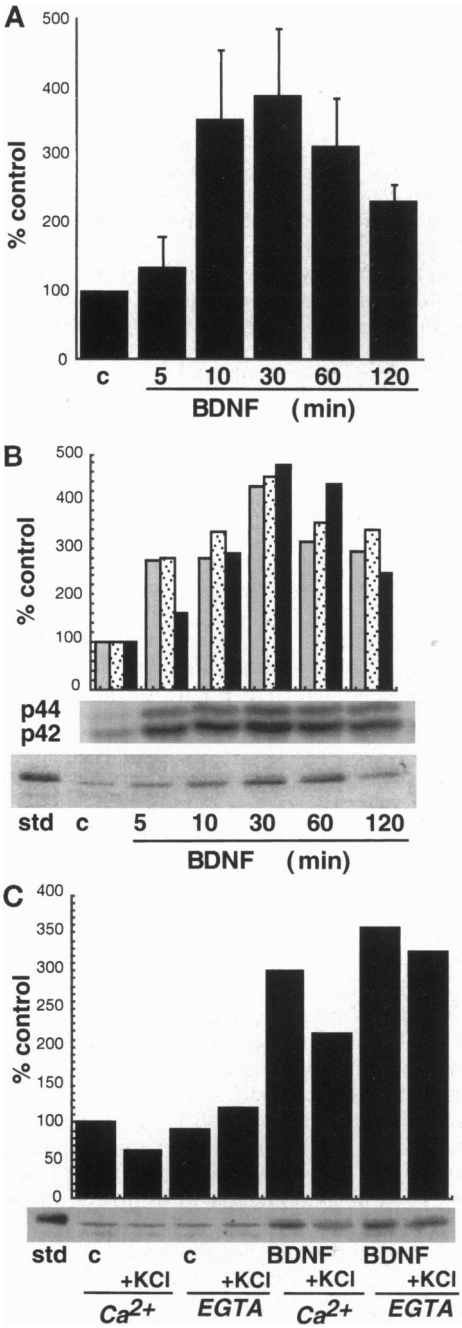

FIG. 3. (*A*) Effect of BDNF (50 ng/ml) on the phosphorylation of synapsin I at sites 4/5 in cerebrocortical neurons (n=4). (*B Top*) Comparison of effects of BDNF on activation of the MAP kinase isoforms p42 (■) and p44 (□) and phosphorylation of synapsin I at sites 4/5 (■). (*B Middle*) Autoradiogram of in-gel MAP kinase assay. (*B Bottom*) Immunoblot analysis of synapsin I phosphorylation using P-site 4/5 Ab. Results are representative of four independent experiments. (*C*) Ca^{2+}-dependent dephosphorylation of synapsin I at sites 4/5. Cultures of cerebrocortical neurons were incubated in the absence (lanes c) or presence (lanes BDNF) of BDNF for 20 min in the presence of 1 mM extracellular Ca^{2+} (lanes *Ca^{2+}*) or Ca^{2+}-free medium containing 0.2 mM EGTA (lanes *EGTA*). Where indicated, samples were depolarized using 60 mM KCl for 1 min (lanes +KCl).

pattern of phosphopeptides (see Fig. 2*B*), which was distinct from those determined previously for other protein kinases (26). Protein microsequencing of purified phosphopeptides revealed two sites, Ser-62 and Ser-67 (referred to as sites 4 and 5, respectively), in the N-terminal "head" region of synapsin I. The third site, Ser-549 (referred to as site 6) was located in the

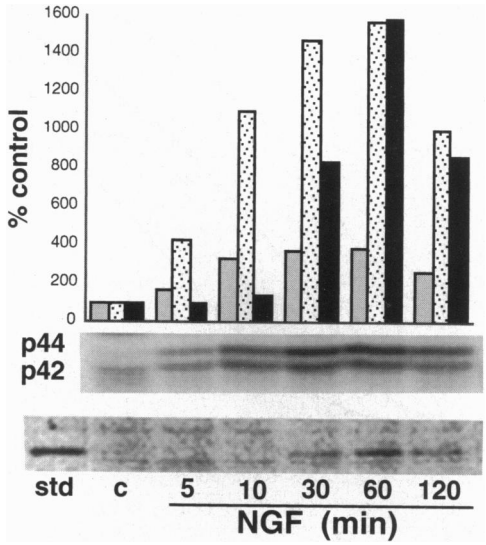

FIG. 4. Effect of NGF (50 ng/ml) on the phosphorylation of synapsin I at sites 4/5 in PC12 cells. (Top) Comparison of effects of NGF on activation of the MAP kinase isoforms p42 (■) and p44 (□) and phosphorylation of synapsin I at sites 4/5 (■). (*Middle*) Autoradiogram of in-gel MAP kinase assay. (*Bottom*) Immunoblot analysis of synapsin I phosphorylation using P-site 4/5 Ab. Results are representative of three independent experiments.

C-terminal "tail" region of the molecule. Site 6 corresponded to the homologous site in bovine synapsin I (Ser-551) shown to be phosphorylated *in vitro* by the cdc2–cyclin A complex (27). The relative location of the six known phosphorylation sites in synapsin I are schematically depicted in Fig. 1*C*.

Synapsin I is Phosphorylated at MAP Kinase-Specific Sites in Intact Preparations. Phosphorylation of synapsin I at sites 4/5 was detected under basal conditions in adult rat cerebral cortex homogenate, and this level appeared to become enriched in purified cerebrocortical synaptosomes (Fig. 2*A Left*). Two major MAP kinase isoforms, p44 and p42, were present in various subcellular fractions, including presynaptic terminals (Fig. 2*A Right*). Basal phosphorylation of synapsin I at sites 4, 5 and 6 in synaptosomes was confirmed by immunoprecipitation of ^{32}P-labeled synapsin I from synaptosomes prelabeled with ^{32}P-orthophosphate. The pattern obtained from two-dimensional phosphopeptide maps revealed nine phosphopeptides (Fig. 2*B Left*), four of which corresponded to the phosphopeptides observed upon in vitro phosphorylation of synapsin I by MAP kinase (Fig. 2*B Right*).

MAP Kinase-Specific Phosphorylation of Synapsin I Is Regulated by Neurotrophins and KCl-Depolarization. The regulation of synapsin I phosphorylation by MAP kinase was examined in primary cultures of rat cerebrocortical neurons. Synapsin I was phosphorylated at sites 4/5 under basal conditions, and this phosphorylation was increased by BDNF (Fig. 3*A*). The effect of BDNF was observed within 5 min, reached a peak of 3.87 ± 1-fold (n = 4) above control levels 30 min after addition and remained elevated over a 2-hr time period. Maximal stimulation of MAP kinase-specific phosphorylation of synapsin I by BDNF was observed at 50 ng/ml (not shown). BDNF-activated MAP kinase isoforms, p42 and p44, with a time course similar to that observed for synapsin I phosphorylation (Fig. 3*B*).

Depolarization of cerebrocortical neurons by 60 mM KCl resulted in a Ca^{2+}-dependent decrease in the phosphorylation state of synapsin I at sites 4/5 (Fig. 3*C*) and site 6 (not shown). Similar effects were observed with synaptosomes (not shown).

In PC12 cells, which express the Trk A receptor, the phosphorylation of synapsin I at sites 4/5 was low under basal conditions. NGF at 50 ng/ml activated the two MAP kinase

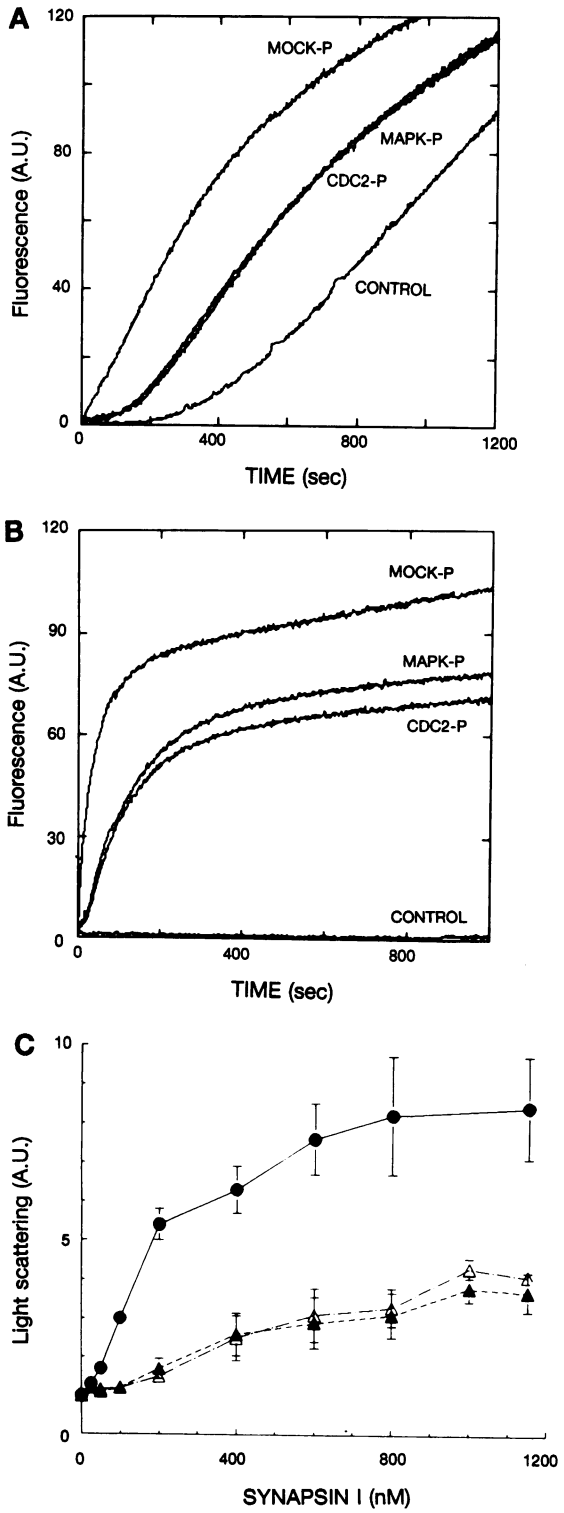

FIG. 5. Ability of various phosphorylated forms of synapsin I to nucleate and polymerize G-actin and bundle F-actin. The synapsin I preparations used were: synapsin I storage buffer alone (CONTROL trace), 300 nM mock-phosphorylated synapsin I (MOCK-P trace), 300 nM synapsin I phosphorylated by MAP kinase (sites 4, 5, and 6) (MAPK-P trace), or 300 nM synapsin I phosphorylated by cdk1 (site 6) (CDC2-P trace). (*A*) Effect of site-specific phosphorylation on the synapsin I-induced acceleration of G-actin polymerization. Polymerization of pyrenyl-G-actin was triggered at time 0 by the addition of KCl and $MgCl_2$ in the presence of the indicated phosphorylated form of synapsin I. Polymerization of pyrenyl-G-actin was analyzed by measuring the fluorescence increase associated with the G-actin–F-actin transition. Experiments were performed under conditions of low ionic strength as described (33). (*B*) Effect of site-specific phosphorylation on synapsin I-induced actin nucleation and polymerization. The polymerization of pyrenyl-G-actin was triggered by the addition at time 0 of the indicated phosphorylated form of synapsin I in the absence of KCl and $MgCl_2$. Experiments were performed under conditions of high ionic strength (33). (*C*) Effects of site-specific phosphorylation on synapsin I-induced F-actin bundle formation. F-actin was incubated with the indicated phosphorylated forms of synapsin I (●, MOCK-P; △, MAPK-P; ▲, CDC2-P) for 30 min at room temperature, and the extent of filament bundling was measured by light scattering. No bundling was seen in the absence of synapsin I. A.U., arbitrary units.

isoforms as well as the MAP kinase-specific phosphorylation of synapsin I (Fig. 4). In a previous study of PC12 cells (34), NGF was observed to stimulate phosphorylation of synapsin I at a novel site(s). On the basis of a comparison of the pattern obtained from two-dimensional phosphopeptide maps, together with the characteristic shift in the electrophoretic mobility of synapsin I, it is now possible to identify the previously unknown protein kinase responsible for the NGF-dependent phosphorylation of synapsin I in PC12 cells as MAP kinase.

Functional Properties of Synapsin I Are Regulated by MAP Kinase-Dependent Phosphorylation. *In vitro*, dephospho-synapsin I promotes the polymerization of G-actin and bundles actin filaments (refs. 18, 32, 35, and 36). The addition of dephospho-synapsin I immediately prior to nucleating salts abolishes the lag phase of polymerization (corresponding to the activation and nucleation of actin monomers), and, in the absence of nucleating salts, induces polymerization (33). Both effects of synapsin I are reduced by phosphorylation at site 1 and abolished by phosphorylation at sites 2 and 3. We therefore determined the effect of MAP kinase phosphorylation of synapsin I on its ability to interact with G-actin. Bovine synapsin I, which had been stoichiometrically phosphorylated by MAP kinase at sites 4, 5, and 6 or by cdk1 at site 6, had a greatly reduced ability to accelerate the kinetics of actin polymerization triggered by K^+ and Mg^{2+} (Fig. 5*A*). When actin polymerization was triggered by the addition of synapsin I in the absence of K^+ and Mg^{2+} under high ionic strength conditions (33), phosphorylation of synapsin I by either MAP kinase or cdk1 markedly decreased its actin-nucleating activity (Fig. 5*B*). It remains to be determined whether the selective phosphorylation of sites 4 and/or 5, located in the N-terminal region of synapsin I, produces a similar effect. Since site 6 is phosphorylated *in vitro* by MAP kinase and cdk1 and also by cdk5 (not shown), further work will be required to determine the relative contribution of these pathways to the *in vivo* phosphorylation of site 6.

The ability of dephospho-synapsin I to bundle actin filaments was reduced by phosphorylation at site 1 and almost abolished by phosphorylation at sites 2 and 3. Dose–response curves for the actin-bundling activities of mock-phosphorylated synapsin I and synapsin I stoichiometrically phosphorylated by either MAP kinase or cdk1 were generated by using a light scattering assay. In comparison with the mock-phospho-form of synapsin I, the MAP kinase-phospho-form and the cdk1-phospho-form exhibited significantly reduced activity (Fig. 5*C*). The level of light scattering was reduced by about 50% at all concentrations tested, without any significant change in the apparent ED_{50} for bundling. Electron microscopy data were consistent with these results (not shown).

In contrast to phosphorylation at sites 2 and 3, phosphorylation of synapsin I at sites 4, 5, and 6 or at site 6 alone did not significantly affect binding to purified synaptic vesicles (not shown).

DISCUSSION

Activation of MAP kinase in response to neurotrophic factors is believed to be critical for differentiation and survival of

various neuronal populations (37–40). Recently, neurotrophins have also been shown to have acute effects on synaptic transmission (41–47). The developmental effects of the neurotrophins are thought to involve transcriptional regulation, while the underlying basis for their effects on synaptic transmission may involve both presynaptic and postsynaptic mechanisms. Our data provide evidence that synapsin I is a physiological substrate for MAP kinase and raise the possibility that alteration of the actin-based cytoskeleton through MAP kinase-dependent phosphorylation of synapsin I might contribute to both the chronic and acute actions of neurotrophins in the central nervous system.

The observation that sites 4, 5, and 6 undergo Ca^{2+}-dependent dephosphorylation upon depolarization, presumably mediated by protein phosphatase 2B (calcineurin), is of interest. Based on this observation, presynaptic entry of Ca^{2+} could have opposing effects on the phosphorylation state of specific sites of synapsin I, increasing phosphorylation at sites 1, 2, and 3, while decreasing phosphorylation at sites 4, 5, and 6. This, in turn, would be expected to have opposing effects on synapsin I-actin interactions. In the present study it was found that neurotrophins are able to enhance phosphorylation of synapsin I at MAP kinase-dependent sites. Since BDNF and neurotrophin-3 have been reported to elevate intracellular Ca^{2+} in hippocampal neurons (48), and since Ca^{2+} can decrease the phosphorylation state of synapsin I at sites 4, 5, and 6 (Fig. 3*C*), the possibility remains open that neurotrophins exert bi-directional control of the phosphorylation of synapsin I at these sites. Finally, further work will be required to determine the contribution, if any, that Ca^{2+}-regulated activation of MAP kinase (5, 6) plays in the regulation of synapsin I phosphorylation by neurotrophins.

The present results demonstrate that neurotrophins and the MAP kinase cascade can be added to the list of signaling pathways that converge at the level of synapsin I. Important subjects to be investigated are (*i*) the elucidation of the complex nature of the temporal and spatial factors that act in concert to modulate the phosphorylation state of synapsin I at at least six known sites and (*ii*) the characterization of the physiological consequences of this phosphorylation in developing and mature neurons.

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