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Phosphorylation of dynamin by ERK2 inhibits the dynamin-microtubule interaction

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Abstract In the present study we show that purified bovine brain dynamin can be phosphorylated by MAP kinase, ERK2, with a stoichiometry of 1 mol phosphate/mol dynamin. The phosphorylated serine residue is located within the C-terminal 10 kDa of dynamin. Dynamin I phosphorylated by ERK2 can be specifically dephosphorylated by calcineurin but not by protein phosphatase 2A (PP2A). Phosphorylation of dynamin by ERK2 weakens the binding of dynamin to microtubules and inhibits dynamin's microtubule-activated GTPase activity. Stimulation of GTPase activity by either Grb2 or phospholipids was not affected by ERK2 phosphorylation, suggesting that the binding sites for Grb2 and phospholipids do not overlap with that for microtubules.

Key words: Dynamin; ERK2; Phosphorylation; GTPase activity

1. Introduction

Dynamins are 90–100 kDa enzymes that catalyze GTP hydrolysis and play an essential, though as yet undetermined, role in synaptic vesicle recycling and receptor-mediated endocytosis [1,2]. Three distinct classes of vertebrate dynamins, dynamins I–III, have been cloned and sequenced [3–5]. All forms of the protein have a tripartite GTP-binding domain near the amino-terminus, a pleckstrin homology (PH) domain that may interact with phosphatidylinositol bisphosphate (PIP_2), and a basic proline-rich tail encompassing the C-terminal 10 kDa. Dynamin I, a neuronal form of the enzyme, is the only form that has been purified to homogeneity and extensively characterized. Several in vitro activators of dynamin I GTPase activity have been identified, including microtubules [6,7], anionic liposomes [8], and Grb2 [9,10], all of which interact with the basic proline-rich tail of the enzyme. Dynamin I is also an in vitro substrate for protein kinases, including protein kinase C (PKC) [11], casein kinase II [11], and cdc2 kinase [12]. Over the last several years, Robinson and his colleagues have demonstrated that dynamin I activity is regulated in vivo by phosphorylation (reviewed in [13]). They showed that dynamin I (also known as dephosphin in earlier studies) is phosphorylated in resting synaptosomes but is rapidly dephosphorylated upon depolarization [14] apparently by the Ca^{2+}/calmodulin-dependent phosphatase calcineurin. Further evidence suggests that PKC is involved in maintaining the phosphorylated state of dynamin in resting neurons, although it has not been proven unequivocally that PKC phosphorylates dynamin in cells. Herein, we demonstrate that bovine brain dynamin I is phosphorylated by the MAP kinase, ERK2. This modification, which can be reversed by calcineurin but not by protein phosphatase 2A (PP2A), results in reduced affinity of dynamin for microtubules and inhibition of its microtubule-activated GTPase activity.

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Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; PKC, protein kinase C; MAP kinase, mitogen-activated protein kinase; MEK1, MAP kinase/ERK kinase; PP2A, protein phosphatase 2A; BSA, bovine serum albumin; GST, glutathione-*S*-transferase

2. Materials and methods

2.1. Materials

Phosphocellulose P11 and diethylaminoethylcellulose DE52 were from Whatman; SP-Sepharose and glutathione-Sepharose were from Pharmacia Biotech.; protease inhibitors and GTP were from Sigma; [γ-^{32}P]GTP was from Amersham; calcineurin was from Boehringer Mannheim; anti-Grb2 antibodies were from Transduction Laboratories. PP2A was a gift from Dr. Marc Mumby (UT Southwestern); anti-dynamin antibodies were provided by Dr. Thomas Südhof (UT Southwestern).

2.2. Purification of proteins

2.2.1. Dynamin purification. Bovine brains were homogenized with 0.1 M MES, pH 7.0, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM DTT, 1 mM sodium azide, and a range of protease inhibitors: 0.2 mM phenylmethylsulfonyl fluoride and 10 mg/liter each of Na-benzoyl-L-arginine methyl ester, Na-*p*-tosyl-L-arginine methyl ester, Na-*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, and pepstatin A (buffer A). The extract was chromatographed on three consecutive ion-exchange columns: DE52 cellulose, P11 phosphocellulose, and SP Sepharose. Fractions highly enriched in dynamin were mixed with microtubules, ultracentrifuged, and dynamin co-sedimenting with microtubules was subsequently released by 10 mM GTP. The supernatant was passed through a DE52 column to remove any traces of tubulin and, if further purification was necessary, was centrifuged on a 5–15% sucrose gradient at $112000 \times g$ for 16 h.

2.2.2. Tubulin. Tubulin was purified according to the procedure of Williams and Lee [15] but MES instead of PIPES buffer was used.

2.2.3. Grb2. Grb2 was expressed in *E. coli* as a fusion protein with glutathione-*S*-transferase (GST) and purified on glutathione-Sepharose.

2.2.4. ERK2. ERK2 was expressed in *E. coli* as described by Xu et al. [16] with a Tev protease cleavage site engineered between the His6 tag and the start of ERK2. Purified ERK2 was treated with Tev protease (Gibco-BRL) following the manufacturer's instructions. ERK2 was activated using constitutively active recombinant MEK1 [17] provided by Dr. Natalie Ahn (HHMI, Univ. Colorado, Boulder). The activation mixture contained 25 mM HEPES, pH 7.5, ERK2 at 0.15 mg/ml, 15% glycerol, 10 μg/ml active MEK1, 1 mM benzamidine, 5 μg/ml leupeptin, 0.5 μM pepstatin, 4 μg/ml aprotinin, 10 mM $MgCl_2$, and 0.3 mM ATPγS. After incubation for 6 h at 30°C, the activated ERK2 was purified on an Ni^{2+}-NTA-agarose column.

2.3. Other methods

2.3.1. Phosphorylation of dynamin. Dynamin was incubated with ERK2 in buffer A containing 10 mM $MgCl_2$ and 50 μM ATP at 30°C and the reaction was terminated by adding EDTA to 20 mM. To determine the extent of phosphorylation [γ-^{32}P]ATP was used (120 cpm/pmol), the reaction was terminated by boiling with SDS, and

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the dynamin band was excised from a Coomassie blue-stained gel and the amount of $^{32}P_i$ was measured by scintillation counting.

2.3.2. Dephosphorylation. ^{32}P-labeled dynamin (0.2 mg/ml) was dialyzed against 25 mM Tris, pH 7.4, 1 mM EDTA, and 1 mM DTT, and then incubated with 0.5 mM ATP and PP2A or 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.5 mM ATP, 10 μg/ml calmodulin and calcineurin for 30 min at 37°C. The phosphatase/dynamin ratio was 1:20 for both PP2A and calcineurin.

2.3.3. Proteolysis. Dynamin (0.2–0.26 mg/ml) was digested with papain for various times at 30°C at a 1:500 enzyme/dynamin ratio. Papain was activated by incubation on ice for 15 min in a solution containing 0.5 M NaCl, 25 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 5 mM DTT. Digestion was stopped by adding iodoacetic acid to a final concentration of 2 mM, then boiling with SDS sample buffer.

2.3.4. Phosphoamino acid analysis. Dynamin phosphorylated with ERK2 (1 mol ^{32}P incorporation/mol) was separated by SDS-PAGE and transferred to Immobilon-P. After staining with Ponceau S red, the band containing dynamin was excised and hydrolyzed in 6 M HCl for 1 h at 110°C. Phosphoamino acids were analyzed by two-dimensional electrophoresis on thin-layer cellulose plates as described by Cooper et al. [18]. Phosphoserine, phosphothreonine, and phosphotyrosine standards were detected by spraying with 0.25% ninhydrin in acetone, and [^{32}P]phosphoamino acids were visualized by autoradiography.

2.3.5. GTPase assays. GTPase activities were measured by the release of $^{32}P_i$ from [γ-^{32}P]GTP [19] after incubation at 37°C in buffer A containing additionally 10 μM taxol, 0.5 mg/ml BSA and 1 mM MgGTP. The reaction time varied from 30 min for low dynamin concentrations to 1 min at high dynamin concentrations to ensure that less than 15% of added GTP was hydrolyzed.

2.3.6. Binding assays. Phosphorylated or unphosphorylated dynamin was mixed with microtubules in the same solution as that used for GTPase activity measurements, incubated for 15 min at 37°C, and then centrifuged at 140000×*g* for 10 min. The pellets were resuspended in the original volume and the amount of dynamin in the supernatants and pellets was determined by scanning SDS-polyacrylamide gel with a Molecular Dynamics Scanner.

2.3.7. Other methods. Protein concentration was determined as described by Bradford [20] using BSA as a standard. SDS-PAGE was carried out according to the method of Laemmli [21] as modified by Matsudaira and Burgess [22]. Immunoblot analysis was carried out by the method of Towbin et al. [23] as described previously [24]. Overlay with GST-Grb2 was according to Feller et al. [25]. Briefly, nitrocellulose was blocked with 2% BSA, preincubated with GST (100 μg/ml) for 6 h and then incubated with GST-Grb2 (10 μg/ml) for 4 h. After washing with blocking buffer, bound Grb2 was identified by immunoblotting with anti-Grb2 monoclonal antibodies.

3. Results

3.1. Phosphorylation of dynamin by ERK2 and characterization of the phosphorylation site

Analysis of its amino acid sequence reveals that dynamin I has 12 potential ERK2 phosphorylation sites that contain serine or threonine followed by proline. Only one of these sites, located near the C-terminus, has the PXS/TP motif usually preferred by this protein kinase. We found that purified dynamin can indeed be phosphorylated by ERK2 up to 1 mol of phosphate/mol of dynamin (Fig. 1A). No significant ^{32}P incorporation was observed in the absence of added kinase (Fig. 1A, inset). Phosphoamino acid analysis shows that serine is the amino acid modified by ERK2 (Fig. 1B). As previously reported [10], limited papain digestion removes from the C-terminus of dynamin a short (about 7 kDa) fragment containing the microtubule-binding site and most of the src-homolog 3 (SH3) binding sites. As expected, the remaining 90 kDa fragment does not express microtubule- or Grb2-stimulated GTPase activities (data not shown). We digested dynamin phosphorylated by ERK2 with papain to localize the radiolabeled phosphorylation site. As shown in Fig. 2, the 90 kDa fragment was not radiolabeled, suggesting that the ERK2 phosphorylation site is within the proline-rich C-terminal domain. To verify that the papain-derived 90 kDa fragment is from the N-terminal portion of dynamin, we showed that intact dynamin but not the 90 kDa fragment can bind to Grb2 (Fig. 2C) and that the 90 kDa fragment is recognized by antibodies raised against a peptide corresponding to residues 607–624 of rat brain dynamin I (Fig. 2D) but not by antibodies against a peptide found in the C-terminus (residues 837–851) (Fig. 2E) (see [4]). Unfortunately, we were unable to

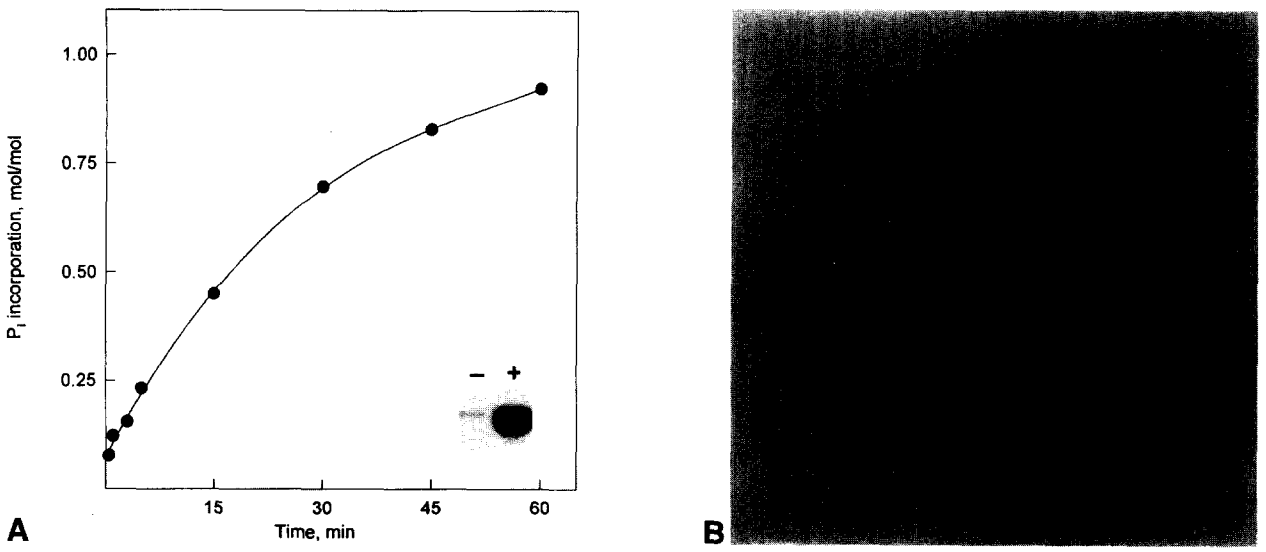

Fig. 1. Phosphorylation of dynamin by ERK. (A) Stoichiometry of phosphorylation. Bovine brain dynamin (3.8 μg) was incubated at 30°C with ERK2 (1 μg) in a reaction mixture (53 μl) containing 100 mM MES, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and 50 μM [γ-^{32}P]ATP (1.2×10^5 cpm/nmol). Reactions were terminated by addition of boiling SDS gel sample buffer and dynamin was purified by SDS-PAGE. (B) Identification of phosphorylated amino acid. Dynamin, phosphorylated as described above, was subjected to HCl hydrolysis, and the amino acids were resolved as described in Section 2.

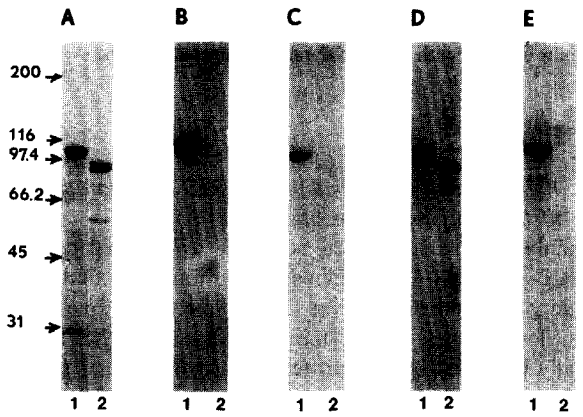

Fig. 2. Localization of the site in dynamin I phosphorylated by ERK2. Dynamin, phosphorylated as described in Fig. 1, was digested with papain (1:500 papain/dynamin ratio) for 10 min at 30°C. Each panel shows intact dynamin in lane 1 and digested dynamin in lane 2. (A) Coomassie blue-stained gel. (B) Autoradiogram. (C) Overlay with GST-Grb2. (D,E) Immunoblots with anti-dynamin antibodies raised against synthetic peptides corresponding to amino acids 607–624 (head domain) or 840–851 (tail domain), respectively.

detect any C-terminal peptides either by autoradiography or by immunoblotting with anti-C terminal antibodies, presumably due to loss of the very small peptides during gel processing.

3.2. *Effect of phosphorylation by ERK2 on the GTPase activity of dynamin*

Tuma and Collins [26] showed that the microtubule-stimulated GTPase activity of dynamin increases cooperatively as a function of dynamin concentration. This result indicates that dynamin self-associates on microtubules and that dynamin-dynamin interactions are involved in GTPase activation. In Fig. 3A, the GTPase activities of phosphorylated and unphosphorylated dynamins, assayed in the presence of 1.6 μM tubulin dimer, are plotted as a function of dynamin concentration. Although phosphorylation does not affect the maximal activity achievable by dynamin at high enzyme concentrations, higher concentrations of phosphorylated dynamin are required for cooperative GTPase stimulation. Half-maximal activation occurs at 0.05 μM dynamin for the unphosphorylated enzyme but at 0.28 μM for the phosphorylated enzyme. Phosphorylation of dynamin did not affect its basal activity which was approx. 5 min^{-1}. This shift in the dynamin dependence curves suggested a reduction in the affinity of dynamin for microtubules due to ERK2 phosphorylation. As determined by a co-sedimentation assay (Fig. 3B), at 0.1 μM dynamin, 91% of unphosphorylated dynamin but only 68% of phosphorylated dynamin is bound to microtubules. However, the amount of phosphorylated dynamin bound to microtubules is still significant while the GTPase activity is very low (10 min^{-1}). At 0.6 μM dynamin, when the GTPase activates are similar for phosphorylated and unphosphorylated enzymes, the amount of phosphorylated or unphosphorylated dynamin bound to microtubules is also similar (69 and 72% of total dynamin, respectively). One can conclude, therefore, that phosphorylation of dynamin by ERK2 reduces the affinity of dynamin for microtubules and also dynamin self-association, especially at low concentration of dynamin. We did not observe any effect of ERK2 phosphorylation on the GTPase activity of dynamin stimulated by either GST-Grb2 or phospholipids (data not shown).

3.3. *Dephosphorylation by calcineurin*

A partially purified dynamin sample (following S-Sepharose chromatography) was used to determine if dynamin phosphorylated by ERK2, like the PKC-phosphorylated enzyme [14], can be dephosphorylated in vitro by calcineurin. This dynamin sample (Fig. 4A) contains an 80 kDa protein which we identified as synapsin by immunoblotting (Fig. 4C, lane 2). Synapsin is also strongly phosphorylated by ERK2 (Fig. 4B, lane 1 and [27]). As shown in Fig. 4B incubation with calcineurin removes the labeled phosphate from dynamin without removing the labeled phosphate from synapsin (Fig. 4B, lane

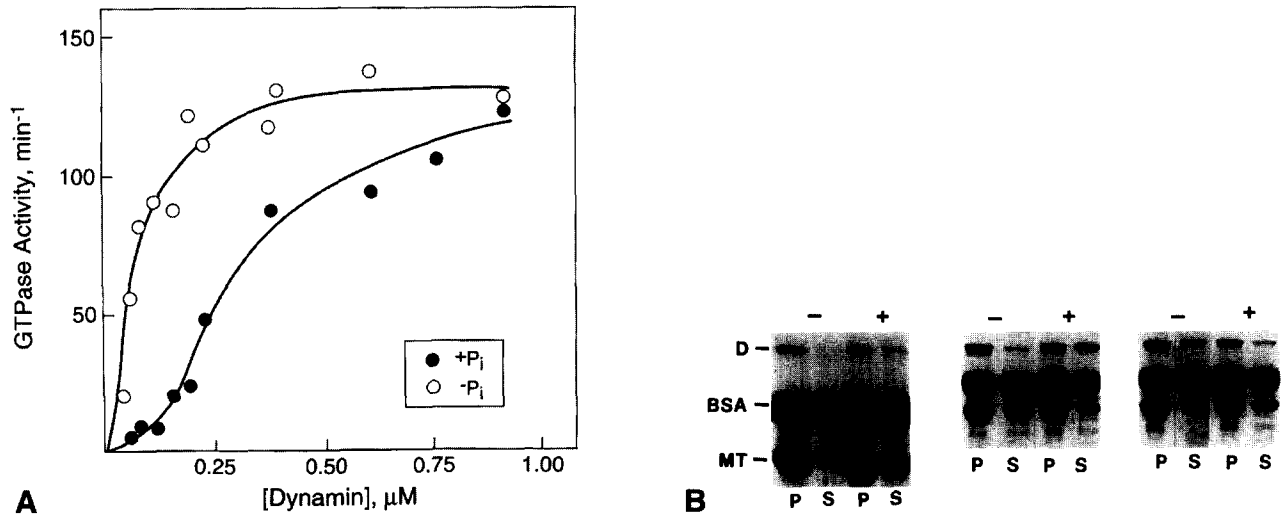

Fig. 3. Effect of dynamin phosphorylation on dynamin-microtubule interactions. (A) Microtubule-activated GTPase activity as a function of dynamin concentration. (B) Binding of unphosphorylation (−) or phosphorylated (+) dynamin to microtubules. p, pellet; s, supernatant. Concentration of tubulin dimer in both A and B was 1.6 μM; dynamin concentrations in B were 0.10, 0.25, and 0.60 μM.

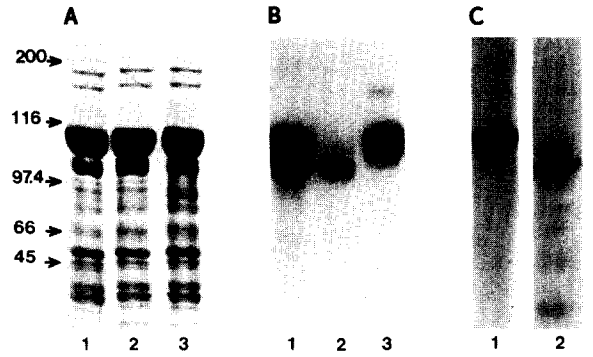

Fig. 4. Dynamin phosphorylated by ERK is dephosphorylated by calcineurin but not by PP2A. A partially purified dynamin preparation that was contaminated with synapsin I was phosphorylated with ERK2 in the presence of [γ-^{32}P]ATP (lane 1), then incubated with either calcineurin (lane 2) or protein phosphatase 2A (lane 3). (A) Coomassie blue-stained gel; (B) corresponding autoradiogram. (C) Immunoblot of the sample using anti-dynamin antibodies (lane 1) or using anti-synapsin antibodies (lane 2).

2). In contrast, protein phosphatase 2A dephosphorylates synapsin but not dynamin (Fig. 4B, lane 3).

4. Discussion

In the present study we show that purified bovine brain dynamin I is a substrate for ERK2, consistent with the presence of consensus phosphorylation sequences found in its basic proline-rich carboxyl terminus. We show that ERK2 phosphorylation indeed occurs in the tail portion of dynamin. Importantly, this modification inhibits dynamin-microtubule interactions. Robinson and his colleagues demonstrated that calcineurin is responsible for dephosphorylation of dynamin in synaptosomes and in vitro when dynamin was phosphorylated by PKC [14]. In this paper, we show dynamin phosphorylated by ERK2 is also dephosphorylated by calcineurin but not by protein phosphatase 2A. In addition, we found that synapsin I, another protein involved in synaptic vesicle trafficking, is a good substrate for ERK2 (Fig. 4, see also [27]) but, in contrast to dynamin, is dephosphorylated by protein phosphatase 2A, but not by calcineurin. These results are in agreement with those published by McPherson et al. [28] who showed that dynamin, but not synapsin I, undergoes dephosphorylation in response to synaptosome depolarization.

Several recent reports have implicated PKC as an important regulator of dynamin I activity in neurons [11,14,29]. The physiological significance of dynamin phosphorylation by ERK2 remains to be determined but our observations indicate that kinases other than PKC may also be involved in the regulation of dynamin activity. The following three considerations support this view: First, the slow time course of dynamin phosphorylation following the termination of depolarization in synaptosomes is consistent with the time course of activation of ERK2 [30]; second, rephosphorylation of dynamin I occurs well after intracellular Ca^{2+} has diminished to resting values, i.e. when cytoplasmic conditions are no longer optimal for PKC activation; and third, there is evidence that dynamin II, the ubiquitous form of the enzyme required for receptor-mediated endocytosis, is not a substrate for PKC [4]. Although our present in vitro experiments were carried out with bovine brain dynamin I, it is likely that our results also apply to the ubiquitously expressed dynamin II. The two forms of dynamin are approx. 70% identical in amino acid sequence and both forms contain several potential ERK phosphorylation sites [4]. Moreover, dynamin I appears to be targeted to the same cellular location as dynamin II since expression of mutant dynamin I in HeLa cells and COS cells (which should not express this isoform) gives a dominant negative phenotype [31,32].

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