Mixed-lineage kinase 2-SH3 domain binds dynamin and greatly enhances activation of GTPase by phospholipid

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Mixed-lineage kinase 2 (MLK2) is a cytoplasmic protein kinase expressed at high levels in mammalian brain. The MLK2 structure is composed of a Src homology 3 (SH3) domain, two leucine zippers, a basic motif, a Cdc42/Rac interactive binding motif and a large C-terminal domain rich in proline, serine and threonine residues. To begin to define the role of MLK2 in mammalian brain, we used an MLK2-SH3 domain–glutathione S-transferase fusion protein (GST–MLK2-SH3) to isolate MLK2-binding proteins from rat brain extract. This analysis revealed that the major MLK2-SH3-domain-binding protein in rat brain is the GTPase dynamin. By using two different forms of the dynamin proline-rich domain as affinity ligands, the binding site for MLK2-SH3 was mapped to the C-terminal region of dynamin between residues 832 and 864. In GTPase assays, the addition of MLK2-SH3 stimulated the activity of purified dynamin I by 3-fold over the basal level, whereas the addition of a known dynamin activator, phosphatidylserine (PtdSer), stimulated a 6-fold increase. When MLK2-SH3 was added to the assay together with PtdSer, however, dynamin GTPase activity accelerated by more than 23-fold over basal level. An MLK2 mutant (MLK2-W59A-SH3), with alanine replacing a conserved tryptophan residue in the SH3 domain consensus motif, had no effect on dynamin activity, either alone or in the presence of PtdSer. In the same assay the SH3 domain from the regulatory subunit of phosphatidylinositol 3′-kinase stimulated a similar synergistic acceleration of dynamin GTPase activity in the presence of PtdSer. These results suggest that synergy between phospholipid and SH3 domain binding might be a general mechanism for the regulation of GTP hydrolysis by dynamin.

INTRODUCTION

The mixed-lineage kinases (MLKs) [1] are a family composed of three closely related protein kinases, MLK1, MLK2 [1,2] and MLK3 [3] and the more distantly related DLK [4]. MLKs 1, 2 and 3 each contain a Src homology 3 (SH3) domain, a kinase catalytic domain, two leucine zippers [5], a basic motif, a Cdc42/Rac interactive binding (CRIB) motif [6] and a large C-terminal domain rich in serine, threonine and proline residues. Within the SH3, kinase, leucine zipper/basic and CRIB domains, MLKs 1, 2 and 3 share a high degree of amino acid similarity. Each of the proteins, however, has a unique N-terminal peptide and, although their C-terminal domains are similar in amino acid composition, they do not share sequence identity. The MLKs also vary in their expression patterns, with MLK2 expressed at highest levels in brain and skeletal muscle [2], whereas MLK3 is expressed abundantly in oesophagus, liver, thyroid, colon and melanoma cells [3]. MLK1 is expressed at low levels in epithelial tumour cells [1], and at higher levels in pancreatic β-cell precursors and during embryonic development [7].

MLK2, MLK3, and DLK have all been implicated in the activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/Jnk) pathway, leading to the phosphorylation of transcription factor c-Jun [8–10]. This pathway transduces signals from stress factors such as osmotic shock, UV irradiation and heat [11], as well as inflammatory cytokines, often leading to cell cycle arrest or apoptosis [12]. MLKs 2 and 3 phosphorylate and activate SAPK–ERK kinase 1/Jnk kinase (SEK1/JNKK) [8,13] and MKK7 [14], direct upstream activators of SAPK/Jnk [15,16]. MLK3 also interacts with the germinal centre kinase [13] and the related haemopoietic progenitor kinase (HPK1) [17]. Furthermore a dominant-negative mutant of MLK3 can block signalling from HPK1 to SEK1/JNKK, suggesting that HPK1 signals through MLK3. In a yeast two-hybrid analysis, proline-rich motifs from HPK1 bound to the SH3 domain of MLK3 [17]. To date, HPK1 is the only protein reported to bind the SH3 domain of a member of the MLK family.

SH3 domains are 55–60-residue globular domains that have been suggested to have important roles in signal transduction, cytoskeletal interactions and control of enzyme activity [18]. Each SH3-domain sequence contains a series of consensus motifs including several highly conserved aromatic amino acids that form a binding pocket for the short proline-rich peptides to which they bind [19]. Because SH3 domains are small and modular, they have often been used as affinity ligands to isolate binding proteins (see, for example, [20,21]). Such an approach was used to identify dynamin as a major binding partner for a number of SH3-domain-containing proteins including c-Src, phospholipase Cγ (PLCγ), GRB2 and the p85 subunit of phosphatidylinositol 3′-kinase (p85-PI3′K) [20].

Dynamin is a 96 kDa GTPase that is involved in vesicle formation during endocytosis, synaptic transmission and secretion [22]. It has three known isoforms: dynamin I, mainly expressed in the brain and nervous system; dynamin II, expressed ubiquitously [23,24]; and dynamin III, primarily expressed in the testis but also found in lung and brain [25]. Dynamin I binds to

Abbreviations used: CRIB motif, Cdc42/Rac interactive binding motif; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; HPK1, haemopoietic progenitor kinase; MLK, mixed-lineage kinase; p85-PI3′K, p85 subunit of phosphatidylinositol 3′-kinase; PRD, proline-rich domain; PtdSer, phosphatidylserine; SAPK/Jnk, stress-activated protein kinase/c-Jun N-terminal kinase; SEK1/JNKK, SAPK–ERK kinase 1/Jnk kinase; SH3, Src homology 3 domain.

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both microtubules [26] and acidic phospholipids [27] and these interactions potently stimulate its GTPase activity. Whereas consensus sequences associated with GTP hydrolysis are located in the N-terminal region of dynamin [28], interactions that regulate GTPase activity have been mapped to the 100-residue C-terminal proline-rich domain (PRD) [29]. The PRD also contains several motifs that have been implicated in SH3-domain binding [29,30]. Furthermore some, but not all, of the SH3 domains that bind dynamin also stimulate its GTPase activity [20].

In the present study, we used a glutathione S-transferase (GST)–MLK2-SH3 domain fusion protein to isolate binding proteins from rat brain. We report here that the major MLK2-SH3-domain-binding protein in rat brain is dynamin GTPase. Furthermore the MLK2 SH3 domain strongly co-operates with phospholipid to effect a significant increase in dynamin GTPase activity over that with lipid alone. This acceleration in GTPase activity by binding of SH3 domains and phospholipids in concert might be a novel mechanism for dynamin regulation.

MATERIALS AND METHODS

Site-directed mutagenesis

The MLK2-W59A mutant was made by using the Altered Sites® II *in vitro* mutagenesis kit (Promega) essentially in accordance with manufacturer's instructions, with the following modifications. *Taq* polymerase (8 units per 30 μl) was substituted for T4 polymerase in the second-strand synthesis reaction and mutant plasmid DNA was transfected into *Escherichia coli* ES1301 *mut*S by electroporation with a Bio-Rad Gene Pulser. For the mutagenesis template, a *Bam*HI fragment containing the first 1488 bp of the coding region of MLK2 cDNA was subcloned into the pALTER-1 vector. The oligonucleotide used to create the MLK2-W59A mutation was 5′-GACGAGGGCTGGGCGACCGGGCAGCT-3′ (altered bases underlined). The entire nucleotide sequence of the mutant insert was determined, to confirm the presence of the desired mutation and the absence of secondary mutations.

Cultured cells and plasmids

The human intraductal breast carcinoma cell line MDA-MB231 was maintained in RPMI-1640 medium supplemented with 10 % (v/v) foetal bovine serum at 37 °C under air/CO_2 (9:1). cDNA species encoding residues 18–78 of MLK2 [2] and MLK2-W59A were amplified by PCR from full-length human wild-type and mutant cDNA species. The sequence of the forward primer was: 5′-CTCGAGTCGACGGATCCCCCGTCTGGACCGCGGTG-3′ and the reverse primer was: 5′-GTCGACTCGAGAATTCTGGGGGCCTCGTAGTTGCT-3′. Recognition sites for the restriction enzymes *Bam*H1 (forward primer) or *Eco*R1 (reverse primer) are underlined in the primer sequences. After digestion with *Bam*H1 and *Eco*R1, cDNA products were ligated into *Bam*H1/*Eco*R1-digested pGEX-KT expression vector and transformed into *E. coli* strain NM522. pGEX-2T constructs encoding either the PRD (residues 751–864) or a truncated form of this domain (residues 751–832) [30] of dynamin Iaa [24] were provided by Dr. P. De Camilli (Yale University, New Haven, CT, U.S.A.); the pGEX-2T plasmid encoding the SH3 domain (residues 2–83) of bovine p85-PI3′K was provided by Dr. Tony Pawson (Mount Sinai Hospital, Toronto, Ontario, Canada).

Production and purification of GST fusion proteins

GST fusion proteins were expressed in *E. coli* and isolated on glutathione–Sepharose beads (Centre for Protein Engineering and Technology, LaTrobe University, Bundoora, Victoria, Australia) by standard methods as described previously [21]. Fusion protein was either stored at 4 °C in 0.01 % sodium azide, eluted from the glutathione–Sepharose with 10 mM reduced glutathione in 20 mM Tris/HCl, pH 7.5, or cleaved with thrombin [1 % (w/w) fusion protein] at 37 °C for 1 h. Eluted or thrombin-cleaved protein was further purified and buffer-exchanged by size-exclusion chromatography on modified methacrylate copolymer, HW-55S (TOSOHAAS, Montgomeryville, PA, U.S.A.). Protein concentrations were determined by using the bicinchoninic acid procedure [31].

Affinity chromatography of rat brain extract

MLK2-SH3 domain proteins were coupled to normal *N*-hydroxysuccinimide-activated Sepharose® 4 Fast Flow (Pharmacia) in accordance with the manufacturer's instructions. In brief, 5 mg of protein in 0.2 M $NaHCO_3$ (pH 8.3)/0.5 M NaCl was incubated with 2 ml of activated Sepharose for 4 h at 4 °C. Uncoupled groups were deactivated by incubation in 0.5 M ethanolamine/0.5 M NaCl (pH 8.3) (high-pH buffer) for 1 h at 4 °C. Columns were then washed alternately in high-pH and low-pH [0.1 M $NaC_2H_3O_2$/0.5 M NaCl (pH 4)] buffers and stored at 4 °C in 10 mM Hepes (pH 7.5)/150 mM NaCl/0.01 % sodium azide. Approximately 4 g of rat brain was homogenized in H buffer [10 mM Hepes (pH 7.5)/150 mM NaCl/10 i.u./ml trasylol/20 μM leupeptin/0.1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride]. Triton X-100 [final concentration 1 % (v/v)] was added and the homogenate clarified by centrifugation (95000 ***g***, 1 h 4 °C). Clarified extract was incubated with either 0.5 ml of GST–MLK2-SH3-Sepharose or GST–MLK2-W59A-SH3-Sepharose for 2 h at 4 °C. For GST–dynamin PRD peptide studies, approx. 100 μl of a 1:1 slurry of MLK2-SH3-Sepharose or MLK2-W59A-SH3-Sepharose was incubated for 2 h at 4 °C with 100 μg of GST–dynamin 751–864, GST–dynamin 751–832 or rat brain extract, in H buffer containing 1 % (v/v) Triton X-100. Columns were washed extensively in H buffer/1 % Triton X-100 and bound proteins were eluted in 100 mM Hepes (pH 6.8)/3 M $MgCl_2$. Proteins were precipitated in 80 % (v/v) methanol at −70 °C for 1 h and recovered by centrifugation (20000 ***g***, 15 min, 4 °C). Protein pellets were freeze-dried, boiled in reducing SDS sample buffer and subjected to SDS/PAGE [10 % (w/v) gel]. Gels were stained with Coomassie Brilliant Blue R250, or proteins were electroblotted to nitrocellulose membrane and probed with monoclonal anti-dynamin (Transduction Laboratories, Lexington, KY, U.S.A.) or anti-GST (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibodies. Antibody complexes were detected with an enhanced chemiluminescence (ECL) detection kit (Amersham).

Affinity chromatography of MDA-MB231 cell lysates

Lysate from 3×10^6 MDA-MB231 cells was subjected to affinity chromatography as above, except that 10 μg of GST, GST–MLK2-W59A-SH3 or GST–MLK2-SH3, bound to glutathione–Sepharose, were used as affinity matrices. After separation by SDS/10 % (w/v) PAGE, bound proteins were electroblotted on to nitrocellulose membrane and probed with anti-dynamin antibodies as above.

Dynamin GTPase assay

Dynamin I was purified from sheep brain as described previously [32] and its GTPase activity was determined as described by Liu et al. [33]. In brief, purified dynamin I (3.7 μg/ml) was incubated in a final volume of 40 μl with 10 mM Tris, pH 7.4, 10 mM

NaCl, 1 mM Mg^{2+}, 0.05 % Tween 80, 1 mM GTP and 2 μCi of [γ-^{32}P]GTP in the presence or absence of sonicated phosphatidylserine (PtdSer) vesicles (20 μg/ml) and SH3-domain fusion proteins for 60 min at 30 °C. The reaction was terminated with 100 μl of acidic solution [2 % (v/v) formic acid/8 % (v/v) acetic acid (pH 1.9)] followed by 600 μl of acid-washed charcoal (10 % [w/v] charcoal in acidic solution) and then 100 μl of BSA (5 mg/ml). After mixing, charcoal was removed by centrifugation and the release of ^{32}P was measured in 200 μl of supernatant by β-counting.

RESULTS AND DISCUSSION

Dynamin is the major MLK2-SH3-domain-binding protein in rat brain and breast tumour cell lysates

SH3-domain interactions are important in the regulation of many cellular processes, often through targeting of proteins to specific subcellular locations or protein complexes. A knowledge of proteins to which particular SH3 domains bind might therefore yield information about the biological systems in which they are found. To identify proteins that bind the SH3 domain of MLK2 in mammalian brain, we employed an MLK2-SH3 domain affinity approach to isolate binding proteins from rat brain. As a control we used an MLK2 mutant (MLK2-W59A-SH3) in which alanine was substituted for a tryptophan residue conserved in the SH3-domain consensus motif. To avoid the large amounts of fusion protein present in glutathione eluates of bound proteins from glutathione–Sepharose columns, the fusion proteins for this experiment were covalently linked to Sepharose beads. Rat brain extract was then subjected to affinity chromatography on columns containing either GST–MLK2-SH3 or GST–MLK2-W59A-SH3 fusion protein. SDS/PAGE analysis of the column eluates (Figure 1a) revealed that a prominent protein of apparent molecular mass < 95 kDa was present in the GST–MLK2-SH3-Sepharose eluate (Figure 1a, lane 2) but not in that from GST–MLK2-W59A-SH3-Sepharose (Figure 1a, lane 1). As

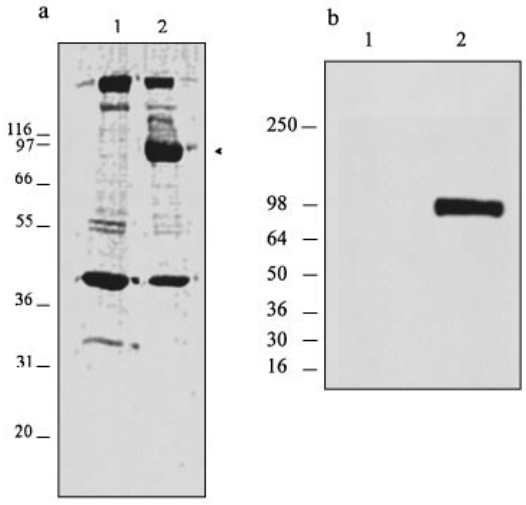

Figure 1 MLK2-SH3 binds dynamin in rat brain extract

(**a**) Coomassie-stained image of SDS/PAGE gel containing proteins from rat brain extract that bound to affinity columns containing GST–MLK2-W59A-SH3-Sepharose (lane 1) or GST–MLK2-SH3-Sepharose (lane 2). (**b**) Proteins isolated from rat brain extract as in (**a**) were electroblotted to nitrocellulose and the membrane was probed with monoclonal anti-dynamin antibodies. Antibody complexes were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the left of each panel.

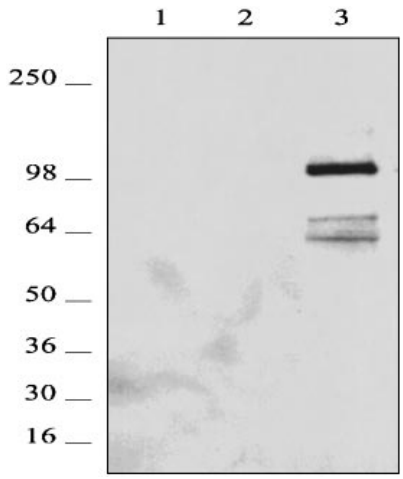

Figure 2 MLK2-SH3 binds dynamin from MDA-MB231 cells

Immunoblot analysis of MDA-MB231 cell proteins eluted from glutathione–Sepharose columns containing GST (lane 1), GST–MLK2-W59A-SH3 (lane 2) or GST–MLK2-SH3 (lane 3). Eluted proteins were separated by SDS/PAGE and electroblotted to nitrocellulose, then the membrane was probed with anti-dynamin antibodies. Antibody complexes were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the left of each panel.

dynamin is an SH3-domain-binding protein of 96 kDa that is abundant in mammalian brain [20,34], we assayed the column eluates by immunoblotting with anti-dynamin antibodies. This analysis (Figure 1b) confirmed the presence of dynamin in the MLK2-SH3 bound fraction (lane 2), and its absence from the MLK2-W59A-SH3 bound fraction (lane 1).

Dynamin I is the major dynamin isoform in mammalian brain [24]. The large amount of dynamin that bound to the MLK2-SH3 affinity column (Figure 1a, lane 2) suggested that MLK2-SH3 binds strongly to dynamin I. To assess whether dynamin from other cell types also binds to the SH3 domain of MLK2, we incubated GST–MLK2-SH3 or GST–MLK2-W59A-SH3 with lysates of cultured MDA-MB231 human breast tumour cells. Eluted proteins were examined by anti-dynamin immunoblotting. From this analysis (Figure 2) it is clear that dynamin from the human breast tumour cell line also binds specifically to MLK2-SH3 (Figure 2, lane 3) but not to MLK2-W59A-SH3 (lane 2) or GST alone (lane 1). The dynamin isoform in human breast tumour cells is unlikely to be dynamin I, as this isoform is expressed mainly in neuronal tissue [23]. This raises the possibility that MLK2-SH3 also binds dynamin II, the isoform that is expressed widely in mammalian tissues [23,24] and is most likely to be present in breast cells.

Although dynamin is a binding partner for several SH3-domain-containing proteins [20], it was unexpected that it should bind to MLK2. MLK family members contain a CRIB motif, found in proteins that bind the small GTPases Cdc42 and Rac, and the MLK CRIB motif binds both of these proteins in a GTP-dependent manner [6]. It might therefore be surprising that a GTPase from a different family should bind through a separate domain. Cdc42 and Rac are involved in changes to the actin cytoskeleton during the formation of filopodia and membrane ruffles respectively in response to extracellular stimuli [35]. Dynamin, in contrast, has a role in synaptic vesicle retrieval and receptor-mediated endocytosis [28,36]. These different GTPase activities, however, are likely to be co-ordinated during cellular activation. Indeed, there is evidence that dynamin is linked to proteins involved with both the actin cytoskeleton [37] and

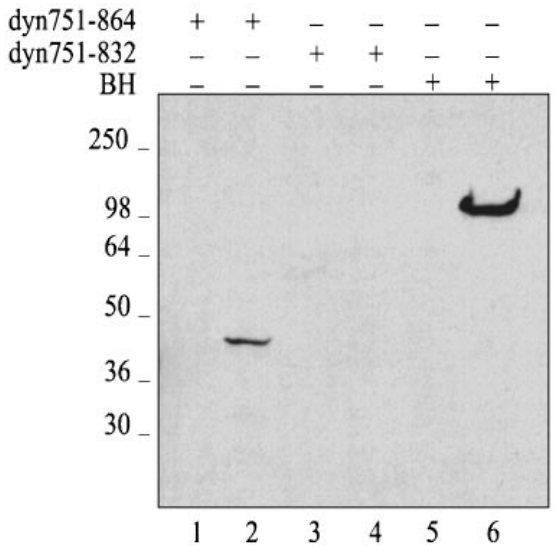

Figure 3 MLK2-SH3 binds dynamin PRD between residues 832 and 864

Immunoblot analysis of fractions from GST–dynamin 751–864 (lanes 1 and 2), GST–dynamin 751–832 (lanes 3 and 4) or rat brain extract (BH) (lanes 5 and 6) eluted from affinity columns of MLK2-W59A-SH3-Sepharose (lanes 1, 3 and 5) or MLK2-SH3-Sepharose (lanes 2, 4 and 6). Eluted proteins were separated by SDS/PAGE and electroblotted to nitrocellulose, then the membrane was split and probed with monoclonal anti-dynamin (top half) or anti-GST (bottom half) antibodies. Antibody complexes were detected by ECL. The positions of molecular mass standards (in kDa) are shown at the left.

phosphoinositide metabolism in nerve terminals [38]. It is therefore possible that MLK2 might be involved in diverse functions within the cell through interaction with different GTPases. Furthermore MLK2 phosphorylates and activates JNKK/SEK1, a major kinase in the pathway that connects cellular stress and inflammation to activation of the transcription factor c-Jun [8]. Whereas Cdc42 and Rac are known regulators of this pathway [39], dynamin has not been implicated in signalling to the stress-activated kinases. The binding of MLK2-SH3 to dynamin therefore suggests a possible new avenue for dynamin interaction with cellular processes through MLK2.

MLK2-SH3 domain binds to proline-rich peptides within the C-terminal regulatory domain of dynamin

The C-terminal 100 residues of dynamin contain acidic phospholipid and microtubule-binding sites [27,29], as well as several proline-rich peptides that conform to consensus sequences for SH3-domain binding [20]. To define which of the proline-rich motifs might be required for binding to the SH3 domain of MLK2, we used two variants of the dynamin C-terminal PRD [30,40] fused to GST. The first contained the entire PRD (residues 751–864) of dynamin I (GST–dynamin 751–864) and the second was truncated at residue 832 (GST–dynamin 751–832) to remove the more C-terminal proline-rich sequences. In this study the MLK2-SH3 and MLK2-W59A-SH3 fusion proteins were cleaved with thrombin to remove GST and then cross-linked to Sepharose beads. MLK2-SH3-Sepharose or MLK2-W59A-SH3-Sepharose was incubated with GST–dynamin 751–832, GST–dynamin 751–864 or rat brain extract. Bound proteins were examined by immunoblot analysis with anti-GST or anti-dynamin antibodies. As can be seen in Figure 3, MLK2-SH3-Sepharose bound GST–dynamin 751–864 (lane 2) and wild-type dynamin from rat brain (lane 6) but not GST–dynamin 751–832 (lane 4). The control MLK2-W59A-SH3-Sepharose, in contrast, failed to bind either of the GST–dynamin PRD fragments or rat brain dynamin. These results suggest that sequences between residues 832 and 864 of dynamin I are required for binding to the MLK2-SH3 domain.

The role of dynamin GTPase in endocytosis and synaptic vesicle transmission has been well established [36] through studies on nerve terminal depolarization [32] and on the *Drosophila* mutant *shibire* [41,42]. *Shibire* flies have a temperature-sensitive mutation in the dynamin I gene that rapidly halts synaptic vesicle endocytosis at the restrictive temperature, leading to paralysis [43,44]. One SH3 domain interaction that seems to be particularly important for dynamin activity in the brain is that with amphiphysin, a synaptic vesicle protein and the major auto-antigen in the paraneoplastic 'stiff-man' syndrome [45]. In a recent study, GST–amphiphysin-SH3 domain injected presynaptically into the lamprey giant reticulospinal synapse inhibited synaptic vesicle recycling and disrupted endocytosis in response to action-potential stimulation [40]. These effects mimic very closely the phenotype observed in *shibire* flies and suggest that the amphiphysin-SH3 domain interaction is vital for dynamin to function in synaptic vesicle retrieval. Interestingly, the amphiphysin-SH3 binding site has been mapped to a dynamin peptide (residues 833–838) within the same region of the PRD as that identified here for the MLK2-SH3 interaction [30]. This site also overlaps a binding site for the double SH3 domain containing adaptor protein GRB2; GRB2 inhibits the binding of radiolabelled amphiphysin to the dynamin PRD [30].

MLK2-SH3 domain and phospholipid binding co-operate to accelerate dynamin GTPase activity

To examine the functional significance of the interaction between MLK2-SH3 and dynamin, we assayed for the stimulation of dynamin GTPase activity by either MLK2-SH3 or MLK2-W59A-SH3. The assays were performed by using purified dynamin I, with or without the acidic phospholipid PtdSer (20 μg/ml) in the reaction mixture (Figure 4). This concentration of PtdSer was previously found to yield optimal stimulation of dynamin GTPase activity in such assays [33]. As a positive control, we used the SH3 domain of p85-PI3′K, which has been shown to stimulate dynamin GTPase activity [20,33]. In the absence of phospholipid, both MLK2-SH3 and p85-PI3′K-SH3 stimulated dynamin GTPase by approx. 3-fold over background (Figure 4a), whereas PtdSer alone stimulated a 6-fold increase in hydrolysis of GTP (0.32 μmol of [^{32}P]P_i released/min per mg). When either MLK2-SH3 or p85-PI3′K-SH3 was added to the assay together with PtdSer, however, the hydrolysis of GTP by dynamin increased by 23-fold over the basal level. Under the same conditions, MLK2-W59A-SH3 had no effect on dynamin activity with or without PtdSer. Although the enhancement of dynamin GTPase stimulation with either MLK2 or p85-PI3′K-SH3-domain proteins reached similar levels (approx. 1.2 μmol/min per mg), a 6-fold higher concentration of GST–p85-PI3′K-SH3 (65 μg/ml) was required to effect the same degree of stimulation as that observed with GST–MLK2-SH3 (10 μg/ml).

To characterize further the activation of dynamin by MLK2-SH3, we measured the dose dependence of activation by MLK2-SH3, with or without PtdSer. In these assays, the concentrations of both dynamin and PtdSer were kept constant while increasing amounts of GST–MLK2-SH3 were added. As can be seen in Figure 4(b), activation by MLK2-SH3 domain, both alone and in synergy with PtdSer, was dependent on the amount of GST–MLK2-SH3. It is interesting to note, however, that a larger amount of MLK2-SH3-domain protein was required

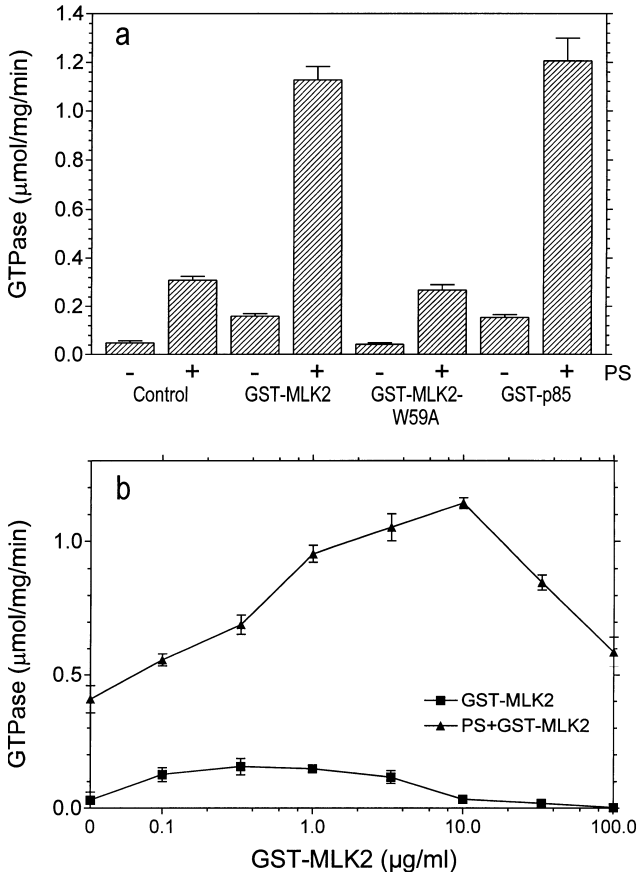

Figure 4 SH3 domains and PtdSer co-operate to activate dynamin I GTPase

(**a**) Purified dynamin I (3.7 μg/ml) was incubated, in the presence or absence of 20 μg/ml PtdSer (PS), in a total volume of 40 μl with GTPase assay buffer alone (control), GST–MLK2-SH3 (10 μg/ml), GST–MLK2-W59A-SH3 (11 μg/ml) or GST–p85-PI3′K-SH3 (65 μg/ml), as indicated. The hydrolysis of [γ-^{32}P]GTP was determined by measuring the release of [^{32}P]P_i, as described previously [41]. (**b**) Assay performed as in (**a**) with increasing amounts of GST–MLK2-SH3, as indicated.

to reach maximal stimulation when it was acting in co-operation with PtdSer. This suggests that PtdSer might be an allosteric regulator of dynamin that allows other activators enhanced access for binding and activation. In earlier reports, several SH3-domain proteins that bind dynamin have been shown to stimulate GTPase activity to varying extents without the addition of phospholipid (see, for example, [20]). As phospholipid can bind constitutively to dynamin in cells, however, residual phospholipid is likely to be present in some dynamin preparations, depending on the purification method. Nevertheless the high level of dynamin stimulation by MLK2-SH3 or p85-PI3′K-SH3 in synergy with PtdSer supports the notion that SH3-domain synergy with phospholipid might be a general mechanism for regulating dynamin activity.

Although the role of the MLK2-SH3 domain in the regulation of dynamin activity *in vivo* is not clear, the strong binding and very high level of GTPase activation in the presence of phospholipid suggest that the MLK2–dynamin interaction is meaningful. In addition, MLK2 is expressed at high levels in most compartments of the brain (results not shown), indicating that the opportunity for interaction *in vivo* is likely. In an earlier study we found that tubulin binds to the N-terminal region of MLK2, close to, but outside, the SH3 domain [46]. Another recent report revealed that MLK2 co-localizes with activated Jnk on microtubules and that the MLK2-C-terminal domain binds KIF3A [47], a microtubule motor protein that is involved in the anteriograde transport of vesicles along microtubules. Interactions with dynamin as well as microtubules and vesicle transport motors suggest that MLK2 might be involved in the regulation of dynamin during vesicle trafficking. Dynamin is a very abundant protein in nerve terminals [34,48], where it interacts with the SH3 domain of amphiphysin to participate in synaptic vesicle transmission [40]. Dynamin GTPase activity is required for fission of the vesicles before release from the cell membrane [48]. To facilitate synaptic vesicle release and/or retrieval, the nerve terminal might require very rapid acceleration of dynamin GTPase activity at the time of synaptic transmission. A requirement for a simultaneous interaction with both SH3 domain and phospholipid to accelerate activity might provide a mechanism for ensuring that dynamin is fully activated only at the exact instant, and at the precise site, that rapid GTPase activity is required. As the MLK2-SH3 domain interaction markedly enhances dynamin activity in the presence of phospholipid, MLK2 might well be involved in the regulation of such a process. It will therefore be of interest to dissect the role of the MLK2–dynamin interaction in both vesicle transport and signal transduction.

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