The *Drosophila* protein kinase LK6 is regulated by ERK and phosphorylates the eukaryotic initiation factor eIF4E *in vivo*

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In *Drosophila* cells, phosphorylation of eIF4E (eukaryotic initiation factor 4E) is required for growth and development. In *Drosophila melanogaster*, LK6 is the closest homologue of mammalian Mnk1 and Mnk2 [MAPK (mitogen-activated protein kinase) signal-integrating kinases 1 and 2 respectively] that phosphorylate mammalian eIF4E. Mnk1 is activated by both mitogen- and stress-activated signalling pathways [ERK (extracellular-signal-regulated kinase) and p38 MAPK], whereas Mnk2 contains a MAPK-binding motif that is selective for ERKs. LK6 possesses a binding motif similar to that in Mnk2. In the present study, we show that LK6 can phosphorylate eIF4E at the physiological site. LK6 activity is increased by the ERK signalling pathway and not by the stress-activated p38 MAPK signalling pathway. Consistent with this, LK6 binds ERK in mammalian cells, and this requires an intact binding motif. LK6 can bind to eIF4G

in mammalian cells, and expression of LK6 increases the phosphorylation of the endogenous eIF4E. In *Drosophila* S2 Schneider cells, LK6 binds the ERK homologue Rolled, but not the p38 MAPK homologue. LK6 phosphorylates *Drosophila* eIF4E *in vitro*. The phosphorylation of endogenous eIF4E in *Drosophila* cells is increased by activation of the ERK pathway but not by arsenite, an activator of p38 MAPK. RNA interference directed against LK6 significantly decreases eIF4E phosphorylation in *Drosophila* cells. These results show that LK6 binds to ERK and is activated by ERK signalling and it is responsible for phosphorylating eIF4E in *Drosophila*.

Key words: *Drosophila melanogaster*, extracellular-signal-regulated kinase (ERK), initiation factor, LK6, protein kinase, translation factor.

INTRODUCTION

The protein kinase LK6 was first identified in a screen using antisera originally raised to microtubule-associated proteins from *Drosophila melanogaster* cells that also recognize centrosomes [1]. The antisera were used to screen the expression libraries to identify microtubule-associated centrosomal proteins. One cDNA identified in this way encodes LK6, which contains, in addition to a canonical protein kinase catalytic domain, an N-terminal extension and a much longer C-terminal region, giving a total molecular mass of approx. 200 kDa [1]. This C-terminal region contains a PEST sequence, which probably accounts for the rapid degradation of LK6. LK6 protein was found to localize (at least in part) to centrosomes and to bind to microtubules. Overexpression of LK6 led to defects in microtubule organization. However, little is known about the regulation, interactions and substrate specificity of LK6.

The catalytic domain of LK6, compared with other human protein kinases, is very similar (61 % identical and 77 % similar residues) to the catalytic domains of Mnk1 and Mnk2 [MAPK (mitogen-activated protein kinase) signal-integrating kinases 1 and 2; see also Figure 1]. These enzymes were first cloned by virtue of their abilities to bind to or be phosphorylated by mammalian ERK (extracellular-signal-regulated kinase; MAPK) [2,3]. Mnk1 and the originally identified splice variant of human Mnk2 (now termed Mnk2a) [4,5] contain a sequence near their C-termini that binds to ERK and, for Mnk1, also to p38 MAPK. Mutation of this motif in the Mnks impairs their ability to bind to ERK and their

activation *in vivo* [5,6]. A second splice variant of Mnk2 (Mnk2b) lacks this MAPK-binding motif and is only poorly activated by ERK [4,5].

Mnk1 and Mnk2a are both primarily cytoplasmic, whereas Mnk2b partially localizes to the nucleus. All three contain a polybasic region near their N-termini that binds importin- α and can mediate the transport of the Mnks to the nucleus [5,6]. Mnk1 contains an LMB (leptomycin B)-sensitive NES (nuclear export signal) that ensures Mnk1 is normally cytoplasmic. Mnk2a and Mnk2b lack this NES but other mechanisms ensure the cytoplasmic localization of Mnk2a, whereas Mnk2b is largely nuclear [5].

The N-terminal polybasic region of the Mnks also mediates their binding to the translation factor eIF4G (eukaryotic initiation factor 4G) [5–7]. eIF4G is a multidomain scaffold protein that interacts with a number of proteins involved in the initiation of mRNA translation [8]. These include eIF4E, the protein that binds to the 5'-cap structure of the mRNA and plays a key role in normal cap-dependent translation initiation [9], and the Mnks [7,10]. The Mnks phosphorylate eIF4E at the physiologically relevant site, Ser²⁰⁹, and are probably the physiological eIF4E kinases in mammalian cells. For example, the level of phosphorylation of eIF4E is regulated through the ERK and p38 MAPK pathways that control Mnk activity [11] and a selective Mnk inhibitor (CGP57380) blocks the phosphorylation of eIF4E in cells [12].

Phosphorylation of eIF4E has been reported to either enhance [13] or impair [14] its binding to cap or capped RNA, and the role of this modification in the control of translation is still

Abbreviations used: CD, catalytic domain; RNAi, RNA interference; dsRNAi, double-stranded RNAi; eIF, eukaryotic initiation factor; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; HEK-293 cells, human embryonic kidney 293 cells; IEF, isoelectric focusing; LMB, leptomycin B; MAPK, mitogen-activated protein kinase; Mnk, MAPK signal-integrating kinase; NES, nuclear export signal; WT, wild-type; for brevity, the single-letter system for amino acids has been used, S209, for example means Ser²⁰⁹.

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Figure 1 Comparison of the Mnks with LK6

The layout and sequence motifs of mouse Mnk1 and Mnk2 and *Drosophila* LK6 are schematically represented. The various domains that are discussed in the text are indicated by differently shaded boxes and the sequences for the nuclear localization signal (NLS), NES and MAPK-binding sites are given in detail. The critical residues forming the NES in Mnk1 and the putative NES in LK6 are shown in boldface, whereas the bold and underlined amino acids indicate the MAPK-binding site. Note that a large part of the C-terminus of LK6 is not shown (indicated by \ \ \).

unclear (see [15] for a review). eIF4E from *Drosophila* contains a serine residue in the position corresponding to Ser²⁰⁹ in mammalian eIF4E and mutation of this residue to an alanine residue completely prevents phosphorylation of eIF4E *in vivo* [16]. Phosphorylation of this site appears to play an important role in normal development and growth in *Drosophila* [16], based on the phenotypes observed when this residue is mutated.

In the light of recent findings concerning the mammalian relatives of LK6, the Mnks, we have studied the regulation, kinase function and selected interactions of LK6. This closest homologue of the Mnks in *Drosophila* phosphorylates mammalian eIF4E *in vitro* and *in vivo* and is activated by ERK signalling but not by the stress-activated p38 MAPK pathway. LK6 also phosphorylates *Drosophila* eIF4E *in vitro*. Detailed studies on functional domains in LK6 reveal substantial similarities, but also important differences, when compared with the mammalian Mnks. The results suggest that LK6 may act as an eIF4E kinase in *Drosophila*.

EXPERIMENTAL

Cell culture and transfection

HEK-293 cells (human embryonic kidney 293 cells) were grown in 10 cm plates in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% (v/v) foetal bovine serum (Invitrogen), 100 units/ml penicillin and 0.1 mg/ml streptomycin. *Drosophila* Schneider S2 cells were grown in 15 cm plates in Schneider's *Drosophila* medium (Invitrogen), supplemented with 10% foetal bovine serum (Invitrogen) at 27°C.

Transient transfections were performed by calcium phosphate precipitation of the DNA in *N,N*-bis-[2-hydroxyethyl]-2-aminoethanesulphonic acid-buffered saline as described earlier [17]. For transfection, 10 and 20 µg of DNA were used per 10 and 15 cm dish respectively. Arsenite (500 µM) or PMA (1 µM) treatment of cells was for 30 min before harvesting. The signalling inhibitors PD98059 and SB203580 (both from Calbiochem) were used for 45 min and at final concentrations of 50 and 10 µM respectively.

Harvesting of cells

After treatment, HEK-293 cells were washed and harvested as described previously [5,6]. Treatments with arsenite, PMA

or signalling inhibitors were as described above for S2 cells. *Drosophila* Schneider S2 cells were washed once with PBS and harvested in 1 ml of buffer A (20 mM Tris/HCl, pH 7.6, 150 mM NaCl, 25 mM β-glycerophosphate, 0.4% Triton X-100, 1 mM dithiothreitol, 0.5 mM sodium orthovanadate, 1 mM PMSF, 1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml antipain and 1 µg/ml pepstatin). Cell debris and nuclei were removed by centrifugation at 14 000 g for 15 min, and the supernatants were transferred to new tubes.

Antibodies

The anti-myc monoclonal antibody (9E10) was obtained from Sigma, p38 antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and anti-ERK antibody was from Cell Signaling Technology (Beverly, MA, U.S.A.). A monoclonal antibody raised against human eIF4E was used as described previously [5]. Other antibodies were gifts from Dr J. M. Sierra (Universidad Autónoma de Madrid, Spain; rabbit anti-*Drosophila* eIF4E), Dr S. Morley (University of Sussex, Brighton, U.K.; rabbit anti-eIF4G and anti-phospho-eIF4E) and Dr L. Zipursky (University of California, Los Angeles, CA, U.S.A.; anti-Rolled antibody).

Expression and purification of recombinant proteins

Human eIF4E was expressed from a pET11d plasmid in *Escherichia coli* BL21(DE3) and purified as described previously [18].

Plasmids

pCS3MT-Mnk1, pEGFP-Mnk1 and pEGFP-Mnk1L378S have been described previously [2,6]. The pCS3MT LK6 and pEGFP LK6 were prepared by cloning a *Bgl*III-*Xho*I- or *Bgl*III-*Kpn*I-digested PCR product (with primers 5'-CGATCAGATCTCCATGGTGGAGCCCCAAGTCC-3' and 5'-GCGGTACCCTCGAGTATCTGTCCACAGTGGACC-3'), encoding the full-length LK6 from pQE90-LK6 (a gift from Dr J. Raff, University of Cambridge, Cambridge, U.K.) [1]. The construct encoding the catalytic domain of LK6 (amino acids 27–427) was prepared by cloning a *Bam*HI-digested PCR product into pCS3MT (the primers used were 5'-CCCCGGATCCATATGACCGAGGTGG-3' and 5'-CCCCGGATCCTAGGCATCCATG-3').

The pCS3MT-*Drosophila* eIF4E was prepared by cloning an *Eco*RI-*Bam*HI-digested PCR product (with primers 5'-ggaattccatgcagagcgacttcacagaatg-3' and 5'-gctctagactacaagtgtagatcgaatttcacg-3'), encoding full-length *Drosophila* eIF4E from pCMV5 *Drosophila* eIF4E (a gift from Dr G. Hernández, Max-Planck-Institut für Biophysikalische Chemie, Abt. Molekulare Biologie, Göttingen, Germany).

All the reactions were performed using the Roche HiFi Expand kit. Mutations in the putative NES (L1202A, Leu¹²⁰² → Ala) or MAPK-binding site (RRAA) were generated using the Quik Change® kit from Stratagene. DNA sequencing was performed by Sequencing Service (School of Life Sciences, University of Dundee, Scotland, U.K.; <http://www.dnaseq.co.uk>) using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

dsRNAi [double-stranded RNAi (RNA interference)]

This was performed as described previously [19,20]. dsRNAi was performed as described previously [20], using LK6₁ (30 µg), LK6₂ (30 µg) and, as a negative control, GFP (green fluorescent protein; 30 µg) dsRNAs per well of a 6-well cell culture dish, with an incubation time of 7 days. Each primer used in the PCR to

generate the dsRNA contained a 5'-T7 RNA-polymerase-binding site preceded by a 5'-GAA overhang (GAATTAATACGACTCACTATAGGGAGA), followed by sense and antisense sequences. For LK6₁, the sense primer CTTCTGCCGCTGCTGCGAAAG and the antisense primer CACGTGCGGCAGTTTTCACC were used. For LK6₂, the same sense primer and a different antisense primer, GTAGAACTTCTTGTCATCC, were employed. For GFP, the sense primer ATGGTGAGCAAGGGCGAGGAGC and the antisense primer TTACTTGTACAGCTCGTCCATGC were used.

Analytical procedures

Immunoprecipitations, kinase assays, gel electrophoresis, Western blotting and IEF (isoelectric focusing) were performed as described previously [5,21,22].

Fluorescence microscopy

HEK-293 cells were grown on coverslips, transfected and images were obtained as described previously [5,6].

RESULTS

LK6 is activated by ERK signalling and phosphorylates eIF4E at Ser²⁰⁹

To study the activity and regulation of LK6, it was important to first establish an assay for it. Bearing in mind the close similarity of its catalytic domain to that of the eIF4E kinases Mnk1 and Mnk2a/b (Figure 1), we tested whether LK6 too could phosphorylate eIF4E. We therefore transfected HEK-293 cells with vectors for myc-tagged LK6^{CD} (where CD stands for catalytic domain, encompassing residues 27–427 of LK6) or, as a control, myc-Mnk1, and 48 h later, we starved the cells of serum overnight. Some plates were then treated with PMA (to activate ERK signalling) or arsenite (to activate p38 MAPK) [11]. Cells were then lysed and the recombinant proteins were immunoprecipitated with anti-myc. Part of the sample was analysed by SDS/PAGE and Western blotting with anti-myc to confirm similar levels of protein expression (Figure 2A). A second aliquot was subjected to a kinase assay using recombinant human eIF4E as substrate. The data presented in Figure 2(A) demonstrate that myc-LK6 phosphorylates eIF4E *in vitro*. The activity of full-length LK6 was tested in a similar manner (Figure 2B) and it also was found to phosphorylate eIF4E. This activity was greatly stimulated by treatment of the cells with PMA (which activates ERK signalling in HEK-293 cells [11]), but not by arsenite, which activates the p38 MAPK pathway. This shows that the ability of LK6 to be activated by ERK is an intrinsic property of the catalytic region of the protein and is not dependent on features outside the catalytic domain, although the degree of activation seen for the full-length protein was greater than that for the catalytic domain. The stimulation of LK6 activity by PMA was largely blocked by the MEK (MAPK/ERK kinase) inhibitor PD98059 [23] (Figure 2B), confirming the involvement of ERK signalling in this process.

To assess whether LK6 phosphorylates eIF4E at the same residue as Mnk1/Mnk2, namely the physiologically relevant site (Ser²⁰⁹), a mutant of eIF4E was used in which Ser²⁰⁹ had been changed to an alanine residue. LK6 was not capable of phosphorylating the resulting S209A variant protein (Figure 2C), indicating that it phosphorylates eIF4E at Ser²⁰⁹ and at no other site, consistent with the situation for the Mnks [2,5]. In this regard, LK6 exhibits a substrate specificity similar to that of Mnk1/Mnk2. It is interesting to note that the amino acid sequence around the phosphorylation site in mammalian eIF4E (KSGSTTK; Ser²⁰⁹ is underlined) shows similarity to the sequence around the cor-

Figure 2 LK6 is activated by ERK signalling

(A) HEK-293 cells were transfected with pCS3MT-Mnk1 or with pCS3MT-LK6^{CD} (which encodes the catalytic domain of LK6, see text) as indicated above the panels and, after 24 h, cells were serum-starved for a further 16 h. Cells were then either untreated or incubated in the presence of PMA or arsenite as indicated. Myc-tagged proteins were immunoprecipitated and an aliquot of the immunoprecipitated protein was analysed by Western blotting (indicated by α -myc). The myc-tagged kinases were tested for their ability to phosphorylate recombinant eIF4E (indicated by 32 P-eIF4E) as described in the Experimental section. (B) HEK-293 cells were transfected with a construct expressing a myc-tagged version of the full-length LK6 protein. After 24 h, cells were serum-starved for 16 h and subsequently harvested directly or treated as indicated with PMA, PMA after preincubation of the cells for PD98059 or arsenite. The expression levels and activity of the expressed LK6 were analysed as described in (A). (C) LK6 phosphorylates the physiologically relevant serine residue in eIF4E. Identical amounts of myc-tagged LK6 from PMA-treated cells (obtained as in B) were incubated with WT recombinant human eIF4E and with recombinant protein in which the serine residue at position 209 has been replaced by an alanine. Phosphorylation of the recombinant proteins was analysed as described in the Experimental section.

responding residue in *Drosophila* eIF4E (**KQGS**SNVK; the equivalent of Ser²⁰⁹ is underlined and identical residues are shown in boldface). This is the physiological phosphorylation site within eIF4E in the fruitfly [16].

As noted above, the activity of LK6 is enhanced by stimulation of ERK but not p38 MAPK (Figure 2B). It is important to note that the putative MAPK-binding site in LK6 conforms to the ERK-binding motif rather than to the motif that can bind both ERK and p38 MAPK (which contains three consecutive arginine residues, [24]). It is therefore more similar to that of Mnk2a compared with Mnk1 (those in LK6 and Mnk2a lack the arginine residue at +2 relative to the first leucine residue; Figure 1).

LK6 binds ERK

To test whether, similar to Mnk1/Mnk2, LK6 can bind stably to ERK, we expressed myc-LK6 in HEK-293 cells, immunoprecipitate it with anti-myc and analysed the pellet by SDS/PAGE/Western blotting using anti-ERK. As shown in Figure 3(A), a clear signal was seen for ERK in the myc-LK6 pull-down but not for pull-downs from cells transfected, as a negative control, with the empty vector.

As mentioned above, LK6 possesses a sequence that is similar to the ERK-binding motif of Mnk2a (and differs slightly from that found in Mnk1; Figures 1 and 3B). To test whether this motif was really responsible for binding ERK, we prepared a mutant of LK6 in which the two arginine residues were mutated to alanine

Figure 3 MAPK binding to LK6

(A) HEK-293 cells were transfected with pCS3MT (empty vector) or with pCS3MT-LK6. The cells were harvested, 24 h after transfection, and the expressed proteins were immunoprecipitated with α -myc antibodies. The presence of ERK was detected by SDS/PAGE and Western blotting with ERK-specific antibodies. (B) The putative MAPK-binding site in LK6: the MAPK-binding sites in the C-termini of Mnk1 and Mnk2 are aligned with the corresponding region in LK6. In mutant LK6(RRAA), the indicated arginine residues (underlined) were replaced by alanine residues. (C) pCSMT-LK6 and pCSMT-LK6(RRAA) were transfected into HEK-293 cells and binding of ERK to the expressed proteins was analysed 24 h after transfection as described in (A). (D) HEK-293 cells were transfected with pCS3MT-LK6 or pCS3MT-LK6 RRAA as indicated above the panels and, 24 h after transfection, were starved of serum for a further 16 h. Cells were then harvested directly (–) or first treated with PMA for 30 min (+). The expressed proteins were immunoprecipitated and their activities against eIF4E were tested as described in the Experimental section.

residues (see Figure 3B; RR/AA mutant). Either this myc-tagged protein or WT (wild-type) myc-LK6 was expressed in HEK-293 cells, immunoprecipitated with anti-myc and pellets were analysed for bound ERK by SDS/PAGE/Western blotting. As shown in Figure 3(C), whereas a clear signal was again seen for ERK in the immunoprecipitate for WT LK6, this was completely lost in the RR/AA mutant. This finding strongly suggests that, as for the Mnks, the LXXRR motif in LK6 functions as the ERK-binding site.

To test the role of this motif in the regulation of the activity of LK6 in cells, we again transfected HEK-293 cells with WT LK6 or the RR/AA mutant and, in some cases, treated the dishes with PMA before lysis, immunoprecipitation and assay of the activity of myc-LK6 against eIF4E. As shown in Figure 3(D), the activity of WT LK6 was significant in the absence of PMA and further increased on the addition of PMA (this is also seen in Figure 2A). Substantial self-phosphorylation of WT LK6 was observed, and this too was increased by PMA (indicated by ^{32}P -LK6). In contrast, no phosphorylation of eIF4E was seen under either condition for the RR/AA mutant. One could argue that this reflects a role for the above motif in substrate binding rather than in regulation: however, a massive loss in the signal for the self-phosphorylation of LK6 itself was also observed, and self-phosphorylation was no longer stimulated by PMA (which is seen for the WT protein; Figure 3D, upper band). The simplest explanation for this is that mutation of the ERK-binding motif abrogates the activation of LK6, leading to a loss of both its autophosphorylation and its activity against eIF4E. Thus the LXXRR-binding motif is required for stable binding to ERK and for activation and regulation of LK6. A similar situation has been observed for the Mnks [5,6]. It should be noted that, in this respect, the full-length protein that cannot bind ERK behaved

Figure 4 LK6 phosphorylates eIF4E in HEK-293 cells and binds to eIF4G

(A) HEK-293 cells were transfected with the indicated constructs. After 24 h, cells were harvested and the presence of myc-tagged Mnk1 and LK6 was analysed by SDS/PAGE and Western blotting with α -myc antibodies (upper panel). Phosphorylation of endogenous eIF4E was determined by purification of eIF4E by chromatography on $m^7\text{GTP}$ -Sephacryl and subsequent analysis by one-dimensional IEF and Western blotting, with detection using anti-eIF4E. The positions of unphosphorylated eIF4E and phosphorylated eIF4E [(p)eIF4E] are indicated on the right of the lower panel. The amount of (p)eIF4E, calculated as a percentage of the total amount of eIF4E, was quantified using ImageJ software (rsb.info.nih.gov/ij/; see [6] for further information). Results are expressed as the means $\pm$ S.E.M. for three completely independent experiments. The increase in eIF4E phosphorylation caused by expression of LK6 or Mnk1 was highly significant ($P < 0.01$) relative to the empty vector controls. (B) HEK-293 cells were transfected with the indicated constructs and the expressed protein were immunoprecipitated with myc-specific antibodies. The amounts of expressed proteins (upper panel; the different proteins are indicated by arrowheads) and the associated eIF4G (lower panel) were assessed by SDS/PAGE and Western blotting with appropriate antibodies (upper section, anti-myc; lower section, anti-eIF4G).

differently from the catalytic domain on its own (which also lacks the ERK-binding site, but can still be activated by PMA treatment (Figure 2A)). It is quite possible that the conformation of full-length LK6 impedes the phosphorylation of its catalytic domain by ERK, and this effect is relieved by deletion of a large (C-terminal) part of that protein. For example, the C-terminus of Mnk2a modulates properties dependent on more N-terminal features of the protein [5]. The observation that ERK signalling can still lead to activation of the catalytic domain of LK6 in the absence of the C-terminal MAPK-binding site is reminiscent of the situation for Mnk2b. Mnk2b contains a C-terminal region that lacks such a site, but is still phosphorylated by ERK, albeit less efficiently when compared with the Mnk2a isoform that does contain an ERK-binding motif [5].

LK6 mediates eIF4E phosphorylation in HEK-293 cells

In view of the facts that LK6 binds to and is apparently activated by ERK in HEK-293 cells and LK6 can phosphorylate eIF4E *in vitro*, we wondered whether it could also increase the phosphorylation of eIF4E within HEK-293 cells. To test this possibility and the probable involvement of the ERK-binding site, we transfected HEK-293 cells with LK6 and then used IEF analysis to examine the level of phosphorylation of the endogenous eIF4E in the cells (after isolating it from cell lysates by affinity chromatography on $m^7\text{GTP}$ -Sephacryl). We also transfected cells with Mnk1 or empty vector (as positive or negative controls respectively).

The level of eIF4E phosphorylation was low in cells that received the empty vector, consistent with previous results [11] (Figure 4A). Expression of Mnk1 resulted in a marked increase in eIF4E phosphorylation as expected. Expression of LK6 also substantially increased the level of eIF4E phosphorylation. Expression levels of Mnk1 and the LK6 proteins were similar, as judged by Western blotting using anti-myc (Figure 4A, upper panel).

Figure 5 LK6 is cytoplasmic and does not shuttle between the cytoplasm and nucleus in an LMB-sensitive manner

(A) HEK-293 cells were transfected with constructs expressing GFP-tagged Mnk1 or LK6 and analysed either directly by fluorescence microscopy (left-hand side panel) or after treatment with 5 ng/ml LMB for 3 h (remaining panels). DAPI (4,6-diamidino-2-phenylindole) staining shows the nuclei, and the merged (GFP + DAPI) images are also shown. Scale bars, 15 μm. (B) HEK-293 cells were transfected with constructs expressing GFP-tagged Mnk1(L378S) or LK6(L1202A). The sequences around the mutated residues are shown on the right: the hydrophobic residues of the known NES (Mnk1) or putative NES (LK6) are shown in boldface and the altered residue is underlined. Cells were fixed 24 h after transfection and then analysed by immunofluorescence as described in the Experimental section.

These results show that LK6 can also act as an eIF4E kinase *in vivo*. To assess whether LK6 could bind eIF4G, it was immunoprecipitated and the pellets were analysed for eIF4G by Western blotting. As shown in Figure 4(B) (lower panel), full-length LK6 did bind eIF4G, but to a lesser extent when compared with Mnk1, which was expressed at a similar level in this experiment (Figure 4B, upper panel). We have shown previously that the binding of eIF4G to human Mnk2a is impaired by features in its C-terminus [5]. It is therefore quite possible that the very long C-terminal region of LK6 also impaired its binding to eIF4G. Hence, we also expressed the N-terminal region of LK6, which contains the catalytic domain and the N-terminal section that includes the polybasic region involved in binding to eIF4G in the Mnk1 [5,6]. This region showed an enhanced ability to interact with eIF4G in the co-immunoprecipitation experiment (Figure 4B), but this was still much lower than that for Mnk1. The lower level of binding of LK6 to eIF4G, in comparison with Mnk1, probably explains the smaller increase in phosphorylation of endogenous eIF4E in cells expressing LK6 when compared with Mnk1 (Figure 4A).

LK6 is cytoplasmic

Thus, although the N-terminus of LK6 does contain a polybasic sequence similar to those found in Mnk1 and Mnk2a/Mnk2b, which allow these kinases to bind eIF4G and importin-α (which mediates transport into the nucleus) [5,6], the sequence in LK6 is less effective than that in Mnk1 in promoting binding to mammalian eIF4G (Figure 4B). Possible reasons for this are discussed below. It was nevertheless of interest to test whether LK6 could be transported to the nucleus. We therefore created a vector for the expression of LK6 as a fusion protein with the GFP in mammalian cells and introduced it in HEK-293 cells. The GFP-LK6 fusion protein showed an exclusively cytoplasmic distribution (Figure 5A) similar to that of GFP-Mnk1 (as reported in [6]), suggesting that it might also contain a potent NES. Inspection of the sequence of LK6 does reveal a possible consensus CRM1-type NES of the pattern $\Phi X_3 \Phi X_2 \Phi X \Phi$, where Φ is a hydrophobic amino acid and X is any residue (Figure 1). Mutation of one of the hydrophobic residues in the corresponding NES of Mnk1 causes the protein to become nuclear ([6] and Figure 5B). However,

mutation of a hydrophobic residue within the putative NES of LK6 (L1202A) had no effect on the localization of the protein, which remained cytoplasmic. It was possible that the long C-terminal region of LK6 contained an additional NES, but treatment of cells with LMB failed to make LK6 nuclear, suggesting that, if it does contain an additional NES, this is not of the LMB-sensitive CRM1 type (Figure 5B).

LK6 binds ERK and is activated by PMA in *Drosophila* S2 cells

The above data show that LK6 can bind to, and be activated by, ERK when expressed in a heterologous system, HEK-293 cells. An important question is therefore whether LK6 also binds the *Drosophila* ERK homologue, Rolled, and whether it can also bind the p38 MAPK homologue. To test this, *Drosophila* S2 ('Schneider') cells were transfected with vectors encoding LK6, Mnk1 (as a control) or with the empty vector. The myc-LK6 or myc-Mnk1 proteins were immunoprecipitated using anti-myc and then analysed for the presence of Rolled or p38 MAPK, together with a blotting using anti-myc to check LK6 expression levels. It is clear from Figure 6(A) that, whereas Mnk1 binds both *Drosophila* p38 MAPK and Rolled, LK6 binds only Rolled. This is consistent both with the presence of different MAPK-binding sites in LK6 and Mnk1 (Figures 1 and 3B) and with the observation that LK6 is only activated by a stimulus that turns on ERK signalling (PMA) and not by one that activates p38 MAPK (arsenite; Figures 2A and 2B). These results also suggest that the different specificities of the LAQRR and LARRR motifs reported for mammalian cells also apply to insect cells; thus, they are probably an evolutionarily conserved feature of MAPK-activated protein kinases.

To assess whether the inability of p38 MAPK to bind to LK6 was also reflected in the regulation of LK6, we tested the abilities of PMA and arsenite to activate LK6 expressed in S2 cells. The data in Figure 6(B) show that PMA led to activation of LK6, whereas arsenite did not; this is entirely consistent with the fact that LK6 binds ERK (Rolled) but not p38 MAPK, and is very similar to the results in Figure 2.

We also analysed the level of phosphorylation of the endogenous eIF4E in S2 cells treated with PMA or arsenite (Figure 6C). eIF4E phosphorylation is already quite high in serum-starved cells, but was further increased by treatment with PMA but not by arsenite treatment. This is again consistent with the properties of LK6 expressed in either S2 or HEK-293 cells. The similarity in the behaviours of the endogenous eIF4E and LK6 is consistent with the notion that LK6 may act as an eIF4E kinase in these cells. Although the compound CGP57380 blocks the activity of the Mnks, we were unable to use it to test the role of LK6 in phosphorylating eIF4E within S2 cells, since it is inactive against LK6 itself *in vitro* (results not shown).

We then wished to study whether LK6 can directly phosphorylate *Drosophila* eIF4E. Although the site corresponding to Ser²⁰⁹ in mammalian eIF4E is present in fly eIF4E, the local sequence shows a number of differences, such that the ability of LK6 to phosphorylate mammalian eIF4E could not be taken as suggesting that it would also phosphorylate the *Drosophila* factor. We initially attempted to address this issue by the co-transfection into S2 cells of vectors for LK6 and *Drosophila* eIF4E, but were repeatedly thwarted by the low efficiency of transfection that can be achieved in these cells. We therefore expressed LK6 and *Drosophila* eIF4E separately in HEK-293 cells and then tested the ability of the former to phosphorylate the latter *in vitro*. As shown in Figure 6(D), both Mnk1 and LK6 were capable of catalysing the phosphorylation of *Drosophila* eIF4E. This demonstrates that LK6 can indeed phosphorylate *Drosophila* eIF4E.

Figure 6 Regulation of LK6 by MAPK signalling in *Drosophila* cells

(A) Schneider S2 cells were transfected with the constructs indicated in the Figure. After 72 h, the cells were harvested and the expressed proteins were immunoprecipitated using α -myc antibodies. Expression of myc-tagged proteins (top panel) and association of p38MAPK (middle panel) or Rolled (*Drosophila* ERK; bottom panel) were analysed by SDS/PAGE and Western blotting with the appropriate antibodies. (B) Schneider S2 cells were transfected with pCS3MT-LK6 and 48 h after transfection the cells were serum-starved for 16 h. The cells were then harvested directly (—) or after treatment with PMA or arsenite as indicated. LK6 was immunoprecipitated with antibodies raised against the myc tag, and the expression levels (indicated by 'LK6') and the activity of LK6 (indicated by 32 P-eIF4E) were analysed as described in the Experimental section. The 'eIF4E input' panel shows that equal amounts of eIF4E were added to each assay. (C) Schneider S2 cells were serum-starved for 16 h and then harvested directly (—) or after treatment with PMA or arsenite as indicated. *Drosophila* eIF4E was purified by m⁷GTP-Sepharose and the phosphorylation state was determined by IEF and Western blotting with antibodies raised against *Drosophila* eIF4E. The positions of unphosphorylated and phosphorylated eIF4E are indicated on the right. (D) HEK-293 cells were transfected with the indicated vectors and treated with PMA after 16 h of serum starvation. Expression levels were assessed by immunoblotting with anti-myc (top panel). After immunoprecipitation with anti-myc, the immunoprecipitates were incubated with recombinant *Drosophila* eIF4E (delf4E) and [γ - 32 P]ATP. The products were resolved by SDS/PAGE and the middle section shows an autoradiograph of the stained gel. The bottom section shows the part of the Coomassie Blue-stained gel containing the delf4E as a 'loading control'. (E) S2 cells, cultured in the presence of serum, were incubated in the presence of LK6₁ or LK6₂ dsRNA or GFP dsRNA, used as negative control, for 7 days as described in the Experimental section. After this period, eIF4E phosphorylation was determined: cells were lysed and eIF4E was isolated by m⁷GTP affinity chromatography, followed by IEF and Western blotting with antibodies raised against *Drosophila* eIF4E. Positions of the phosphorylated and non-phosphorylated forms of eIF4E are shown. Numbers below each lane indicate the percentage of eIF4E in the phosphorylated form as determined by densitometry (as described for Figure 4).

Lastly, we considered it important to address the key issue, whether LK6 actually mediates eIF4E phosphorylation *in vivo*. Extensive use has been made of RNAi to study signalling processes in *Drosophila* S2 cells (see e.g. [19,25]). We therefore prepared the vectors LK6₁ and LK6₂ to generate dsRNAs for RNAi, and also prepared a vector to make GFP dsRNA molecules as a negative control. The level of eIF4E phosphorylation was then analysed by IEF/Western blotting as was done for Figure 6(C). As shown in Figure 6(E), the level of eIF4E phosphorylation in cells receiving the LK6₁ or LK6₂ dsRNA was markedly decreased relative to the cells receiving the GFP dsRNA, used as negative control here in the same way as in [19]. The available antisera for LK6 detect a multiplicity of bands in Western-blot analyses [1] and we were unable to use this approach to assess the efficiency of the 'knockdown' of LK6 expression that we had achieved.

DISCUSSION

This study is the first to provide information on the properties and regulation of the *Drosophila* protein kinase LK6. Its catalytic domain is strikingly similar to those of mammalian Mnks; similar to them, in mammalian cells LK6 can bind to ERK, can be activated by ERK signalling and can phosphorylate eIF4E. This occurs at the physiological site, Ser²⁰⁹. The MAPK-binding motif of LK6 is of the type previously shown to bind ERK but not p38 MAPK [2,3,24,26–28]. Consistent with this, when expressed in mammalian cells, LK6 is not activated by stimuli that turn on p38 MAPK.

It is more challenging to perform similar experiments in *Drosophila* cells owing to the difficulty in transfecting, e.g. S2 cells with high efficiency. However, importantly, we show that LK6 also interacts with the ERK homologue Rolled, but not with the *Drosophila* p38 homologue. Our results, furthermore, show that LK6 is activated by PMA, but not by arsenite, which activates p38 MAPK. The regulatory properties of LK6 thus appear to be similar in mammalian and *Drosophila* cells, indicating that the specificity of the MAPK-interaction motifs is probably similar in both mammals and Diptera. Similar to Mnk1 and Mnk2a, LK6 is primarily, if not exclusively, cytoplasmic. It does contain a basic region of the type that, in Mnk1 and Mnk2, can bind to the nuclear shuttling protein importin- α . It therefore seems probable that either (i) it contains an NES, which ensures its efficient re-export from the nucleus, or (ii) the basic region is not accessible to importin- α . The lack of effect of LMB on the localization of LK6 rules out the operation of a CRM1-type NES of the kind found in Mnk1, although the very long C-terminal extension of LK6 might contain an LMB-insensitive NES.

By analogy with the Mnks [5,6], it is probable that the N-terminal polybasic region of LK6 mediates its binding to eIF4G and could also interact with importin- α . Given that full-length LK6 shows less efficient binding to eIF4G when compared with Mnk1, it also seems possible that it binds importin- α less efficiently, which may contribute to the finding that LK6 is cytoplasmic. We have shown previously [5] that even the much shorter C-terminus of Mnk2a impedes access to the N-terminal basic region in that protein, so it is entirely possible that the much larger C-terminal part of LK6 has a similar effect. This could explain why the fragment of LK6 that lacks the C-terminus bound better to eIF4G than did the full-length protein. It may also be that the low degree of binding reflects the fact that we were studying the association of LK6 with the heterologous human protein, rather than with *Drosophila* eIF4G. We have repeatedly tried to use the available antisera to examine the association of LK6 with eIF4G in S2 cells, but without success. Comparison of the polybasic region of LK6 with those of Mnk1 and Mnk2a (which do bind eIF4G and importin- α [5–7]; Figure 1), and our recent results for mutants with alterations in these features, do not reveal any difference that might obviously explain the decreased ability of LK6 to bind mammalian eIF4G.

As argued above, the C-terminus of LK6 may also impair its activation by ERK, based on the observation that the catalytic domain is more effectively activated than a mutant of the full-length protein that also lacks the ERK-binding motif.

Our results support the idea that LK6 is a *Drosophila* eIF4E kinase. LK6 can phosphorylate eIF4E *in vitro* and its overexpression in cells leads to increased phosphorylation of endogenous eIF4E. Furthermore, the activation of LK6 by ERK signalling but not by p38 MAPK signalling correlates well with the observed behaviour of the phosphorylation of eIF4E in PMA- or arsenite-treated *Drosophila* cells, and the fact that LK6 is activated by stimuli that stimulate ERK but is not activated by stimuli that

activate p38 MAPK, in HEK-293 cells. The ability of LK6 to bind eIF4G also supports the contention that it can act as an eIF4E kinase *in vivo*.

The observation that phosphorylation of the endogenous eIF4E in S2 cells is increased by PMA but not by arsenite is consistent with the regulatory properties of LK6 and with the notion that LK6 may phosphorylate eIF4E in these cells. The fact that it is the only close homologue of the Mnks in the fruitfly genome is also consistent with this notion. Phosphorylation of eIF4E has previously been shown to play an important role in growth in this organism and in its normal development [16]. Our data show that LK6 can phosphorylate *Drosophila* eIF4E *in vitro*, consistent with the idea that LK6 acts as an eIF4E kinase in this organism. The dsRNAi data that we have obtained, which show that two different interfering dsRNAs directed against LK6 each markedly decrease eIF4E phosphorylation in S2 cells, offer strong support to the conclusion that LK6 acts as an eIF4E kinase in *Drosophila*. Unfortunately, the poor quality of the available anti-LK6 antisera prevented us from being able to assess whether the incomplete nature of the loss of phosphorylation of eIF4E reflects incomplete elimination of LK6 expression.

Previous genetic studies have linked LK6 to Ras signalling in *Drosophila* [29]. This agrees very well with our finding that LK6 is activated by ERK signalling, since ERK lies downstream of Ras. LK6 was first identified as interacting with microtubules and centrosomes [1]. Overexpression of LK6 led to defects in microtubule organization, indicative of their increased stability. The connections between the phosphorylations of eIF4E and microtubules are not immediately obvious. However, it is entirely possible that LK6 has additional substrates that interact with microtubules or are components of centrosomes and their phosphorylation may be important in the regulation of, for example, mitosis. Numerous microtubule-associated proteins are indeed phosphorylated. Microtubules undergo massive reorganization during mitosis and this involves an array of phosphorylation events and protein kinases [30]. It may therefore be relevant that LK6 is activated by mitogenic signalling (i.e. through ERK and thus Ras).

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