# Regulation of the polarity kinases PAR-1/MARK by 14-3-3 interaction and phosphorylation

**Olga Göransson[1,*,‡], Maria Deak[1], Stephan Wullschleger[1], Nick A. Morrice[1], Alan R. Prescott[2] and Dario R. Alessi[1]**

[1]University of Dundee, MRC Protein Phosphorylation Unit, James Black Centre and [2]University of Dundee, Division of Cell Biology and Immunology, MSI/WTB complex, Dow Street, Dundee, DD1 5EH, Scotland, UK

*Present address: Lund University, BMC, C11, S-22184 Lund, Sweden
‡Author for correspondence (e-mail: Olga.Goransson@med.lu.se)

*Accepted 12 June 2006*
*Journal of Cell Science 119, 4059-4070 Published by The Company of Biologists 2006*
*doi:10.1242/jcs.03097*

## Summary

**Members of the PAR-1/MARK kinase family play critical roles in polarity and cell cycle control and are regulated by 14-3-3 scaffolding proteins, as well as the LKB1 tumour suppressor kinase and atypical protein kinase C (PKC). In this study, we initially investigated the mechanism underlying the interaction of mammalian MARK3 with 14-3-3. We demonstrate that 14-3-3 binding to MARK3 is dependent on phosphorylation, and necessitates the phosphate-binding pocket of 14-3-3. We found that interaction with 14-3-3 was not mediated by the previously characterised MARK3 phosphorylation sites, which led us to identify 15 novel sites of phosphorylation. Single point mutation of these sites, as well as the previously identified LKB1- (T211) and the atypical PKC sites (T564/S619), did not disrupt 14-3-3 binding. However, a mutant in which all 17 phosphorylation sites had been converted to alanine residues (termed 17A-MARK3), was no longer able to bind 14-3-3. Wild-type MARK3 was present in both the cytoplasm and plasma membrane, whereas the 17A-MARK3 mutant was strikingly localised at the plasma membrane. We provide data indicating that the membrane localisation of MARK3 required a highly conserved C-terminal domain, which has been termed kinase-associated domain-1 (KA-1). We also show that dissociation of 14-3-3 from MARK3 did not affect catalytic activity, and that a MARK3 mutant, which could not interact with 14-3-3, was normally active. Finally, we establish that there are significant differences in the subcellular localisation of MARK isoforms, as well as in the impact that atypical PKC overexpression has on 14-3-3 binding and localisation. Collectively, these results indicate that 14-3-3 binding to MARK isoforms is mediated by multiple phosphorylation sites, and serves to anchor MARK isoforms in the cytoplasm.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/19/4059/DC1

Key words: PAR-1/MARK, 14-3-3, Cell polarity, Phosphorylation site

## Introduction

Members of the PAR-1/MARK (partition-defective or microtubule-affinity regulating) kinase family are conserved from yeast to humans, and have been shown to play crucial roles in cellular functions such as polarity and cell-cycle control (reviewed in Drewes, 2004; Tassan and Le Goff, 2004). PAR-1 was first isolated in *C. elegans*, as one of six *Par* genes required for the formation of anterior-posterior asymetry of the nematode embryo (Guo and Kemphues, 1995; Kemphues et al., 1988; Pellettieri and Seydoux, 2002). PAR-1 homologues have subsequently been identified and studied in a number of organisms, including yeast, *Drosophila* and mammals (Drewes et al., 1997; Elbert et al., 2005; La Carbona et al., 2004; Shulman et al., 2000; Trinczek et al., 2004). These studies have further implicated a role for PAR-1 in regulating cell polarity and other cellular functions, such as mitogenic signalling and cell-cycle control.

Human PAR-1 is encoded by four genes, giving rise to the isoforms MARK1 (PAR-1c), MARK2 (PAR-1b/EMK), MARK3 (PAR-1a/p78/C-TAK1) and MARK4 (PAR-1d/MARKL1) (Tassan and Le Goff, 2004). As in *C. elegans* and *Drosophila*, human MARK (hMARK) isoforms are asymmetrically localised in epithelial cells (Bohm et al., 1997). Furthermore, MARK1 and MARK2 was required for normal polarisation of kidney- (MDCK cells) or liver epithelium (Bohm et al., 1997; Cohen et al., 2004), and for neurite outgrowth (Biernat et al., 2002; Brown et al., 1999). Expression of MARK4 was upregulated in glioblastomas, as well as in hepatocellular carcinomas, suggesting a role for MARK4 in tumorigenesis (Beghini et al., 2003; Kato et al., 2001). Furthermore, MARK4 expression was also induced during focal cerebral ischemia, and cell viability of neuronal cells was decreased following the overexpression of MARK4 (Schneider et al., 2004).

Mammalian PAR-1 was first purified from brain, and named microtubule affinity regulating kinase (MARK), based on its ability to phosphorylate microtubule associated proteins (MAPs) such as MAP2, MAP4 and tau, resulting in their dissociation from microtubules (Drewes et al., 1997; Trinczek et al., 2004). The residue in tau phosphorylated by MARK isoforms, S262, is hyperphosphorylated in Alzheimer's disease. Genetic analysis in *Drosophila* indicated that phosphorylation of this residue primes the hyperphosphorylation of tau by other kinases (Nishimura et al., 2004).

Another emerging action for the MARK isoforms is to control the interaction of their substrates with 14-3-3 adaptor proteins. 14-3-3 proteins interact with discrete phospho-Ser or phospho-Thr motifs in a large number of proteins and, in this way, modulate diverse cellular processes (Mackintosh, 2004). Interestingly, 14-3-3 proteins are themselves members of the *Par* family (PAR-5) (Kemphues et al., 1988), and have been linked to tau hyperphosphorylation and formation of neurofibrillary tangles in Alzheimer's disease (Hashiguchi et al., 2000; Layfield et al., 1996). The first 14-3-3-binding MARK substrate to be identified was the Cdc25c phosphatase, which dephosphorylates, and thereby activates, the CDC2/cyclinB complex, an event that is required for entry into mitosis (Sebastian et al., 1993). In non-mitotic cells, Cdc25c is sequestered in the cytoplasm through MARK3-dependent phosphorylation and subsequent binding to 14-3-3 (Peng et al., 1998). Other substrates, which all bind 14-3-3 as a result of phosphorylation by MARK3, include the kinase suppressor of Raf-1 (KSR1), which functions as a docking platform for components of the Ras-MAPK pathway (Muller et al., 2001), Protein-tyrosine phosphatase H1, which regulates cell-cycle progression and attenuates T-cell-receptor signalling (Zhang, S. H. et al., 1997), and plakophilin2, a desmosomal protein (Muller et al., 2003). A common effect of the MARK3-induced binding to 14-3-3 might be to prevent entry into mitosis by sequestering these proteins in the cytoplasm. The mechanism of action described above also extends to *Drosophila*, in which the PAR-3 protein Bazooka is phosphorylated by *Drosophila* PAR-1 (dPAR-1), inducing its binding to d14-3-3 (Leo), and its dissociation from PAR-6 and atypical protein kinase C (aPKC) (Benton and St Johnston, 2003). The PAR-3–PAR-6–aPKC complex is required for the regulation of polarity in both *C. elegans* and *Drosophila* (Pellettieri and Seydoux, 2002).

One challenge is to elucidate how MARK itself is regulated. MARK isoforms are members of the AMP-activated protein kinase (AMPK) family of kinases and share a similar domain structure, possessing a highly conserved N-terminally located catalytic domain, followed by a ubiquitin-associated domain (UBA), a more diverse spacer region, and a conserved, so called, kinase associated domain (KA)-1 of unknown function at their C-terminus (Drewes, 2004). MARK isoforms, as well as other AMPK family kinases, are activated by phosphorylation of the T-loop on a threonine residue. This phosphorylation is carried out by the tumour suppressor kinase LKB1 (Alessi et al., 2006; Lizcano et al., 2004). In addition, a TAO-1 like kinase purified from brain, termed MARKK, was also reported to phosphorylate the T-loop of MARK isoforms (Timm et al., 2003). MARK2 and MARK3 (Hurov et al., 2004), as well as *Xenopus* PAR-1b (xPAR-1b) (Kusakabe and Nishida, 2004), are also phosphorylated by aPKC. This phosphorylation occurs on one or two sites, depending on isoform and species, located in the spacer region.

MARK isoforms are constitutively active in cells (Lizcano et al., 2004) and little is known about the regulation of these enzymes at the level of subcellular localisation and/or interacting proteins. Intriguingly, not only do MARK isoforms induce binding of their substrates to 14-3-3, but they have also themselves been demonstrated to bind 14-3-3 (Al-Hakim et al., 2005; Benton et al., 2002; Brajenovic et al., 2004; Jin et al., 2004; Kusakabe and Nishida, 2004). The mechanism by which 14-3-3 binding to MARK isoforms is mediated, and how it controls MARK activity and localisation, is not yet understood. This could provide an important clue to how MARK function is regulated and is the focus of this study.

## Results

### Previously characterised phosphorylation sites are not required for binding of MARK3 to 14-3-3

We initially focused our study on MARK3, as co-operation with 14-3-3 to regulate substrates has mainly been described for this isoform. MARK3 is phosphorylated at T211 by LKB1 (Lizcano et al., 2004) and on T564 by aPKC (Hurov et al., 2004). In xPAR-1b (equivalent to hMARK2), aPKC phosphorylated T564 as well as a second C-terminal site (equivalent to S619 in hMARK3) (Kusakabe and Nishida, 2004). To investigate whether the LKB1 and aPKC phosphorylation sites are required for binding of 14-3-3 to hMARK3 (hereafter referred to as MARK3), Ala-mutants of these residues were expressed as glutathione S-transferase (GST)-fusion proteins in HEK 293 cells, and monitored for their ability to interact with endogenous 14-3-3 isoforms. As shown in Fig. 1, wild-type MARK3 bound to 14-3-3ζ and 14-3-3ϵ, as determined by mass-fingerprinting (Fig. 1A), and this interaction was not disrupted by mutating the aPKC sites T564 and S619, alone or in combination (Fig. 1B). Mutation of T211, or of the catalytic residue D196, resulted in only a modest decrease in the ability of MARK3 to bind 14-3-3. In dPAR-1, a fragment encompassing the kinase- and UBA-domain reportedly bound 14-3-3 (Benton et al., 2002). However, an equivalently truncated version of MARK3 (termed kd+UBA) failed to bind 14-3-3 (Fig. 1B). As expected, mutation of T211 or D196 resulted in the loss of kinase activity, whereas mutation of T564 and/or S619 in MARK3 did not significantly affect T-loop phosphorylation (assessed using an antibody specific for phosphorylated T211) or kinase activity, monitored by employing the AMARA peptide substrate or the Cdc25c protein substrate.

### Binding of MARK3 to 14-3-3 requires phosphorylation and an intact phospho-Ser/Thr binding pocket of 14-3-3

Based on the study of three 14-3-3 point mutations and their ability to bind dPAR-1, Benton et al. suggested that the interaction of dPAR-1 with 14-3-3 is independent of phosphorylation (Benton et al., 2002). We next examined the requirement of phosphorylation for the binding of MARK3 to 14-3-3. Treatment of GST-MARK3, purified from HEK 293 cells, with phosphatase in vitro, resulted in dephosphorylation of MARK3 as seen by increased electrophoretic mobility (Fig. 2A, upper panels) and a near ablation of T-loop phosphorylation. The ability to bind either recombinant 14-3-3 in an overlay assay, or the co-purified endogenous 14-3-3, was completely lost as a result of phosphatase treatment. Furthermore, we found that washing GST-MARK3 while still bound to glutathione-Sepharose, with a phosphopeptide but not a dephosphopeptide derived from the 14-3-3-binding sequence in Raf, resulted in the dissociation of 14-3-3 from MARK3 (Fig. 2B). The removal of 14-3-3 from MARK3 did not affect the kinase activity, as measured using the AMARA-peptide- or the Cdc25c protein-substrate.

X-ray crystallographic analysis of 14-3-3 complexed to phosphopeptides, has revealed an amphipathic pocket in

which phosphopeptides, as well as 14-3-3-binding phosphoproteins, such as Raf, have been shown to dock (Liu et al., 1995; Rittinger et al., 1999, Petosa, 1998). The phospho-Ser/Thr binding pocket in 14-3-3 is largely formed by four α-helices (Fig. 2C, upper panels), two of which contain a stretch of basic residues (helices 3 and 5, turquoise and green respectively) and two that contain hydrophobic residues (helices 7 and 9, red and orange respectively). Several of these residues have previously been demonstrated to be crucial for the interaction of 14-3-3 with phosphorylated or non-phosphorylated proteins, such as Raf and the *Pseudomonas* protein exoenzyme S (Exo S) (Wang et al., 1998; Zhang, L. et al., 1997; Zhang et al., 1999). To learn in more detail about the nature of MARK3 binding to 14-3-3, we generated a number a 14-3-3ζ mutants, in which basic residues in helix 3 and 5 had been converted to Glu, and hydrophobic residues in helix 7 and 9 to charged amino acids. We also included three point mutations, which have previously been described as inhibitors of Ras-Raf signalling (Chang and Rubin, 1997), E180K, F196Y and Y211F, of which the two latter are located outside of the amphipathic groove (Fig. 2C, upper panels). These mutants were investigated for their ability to bind endogenous MARK3, Raf and the AMPK-related kinase QSK. As demonstrated in Fig. 2C (lower panels), the integrity of the amphipathic groove was found to be crucial for MARK3 binding, as the majority of mutants, in particular the ones targeting basic amino acids, failed to bind MARK3. Interestingly, although not part of the groove itself, mutation of F196 and Y211 also led to a significant decrease in MARK3 binding. Some residues, such as K49, R56, K120, Y128 and L176, were found to be essential for binding MARK3 as well as Raf and QSK. Other residues, such as R127, I217 and W228 were required for MARK3 and QSK binding, but not for Raf binding an conversely, mutants of E180 or L220 failed to bind Raf, but retained some ability to interact with MARK3.

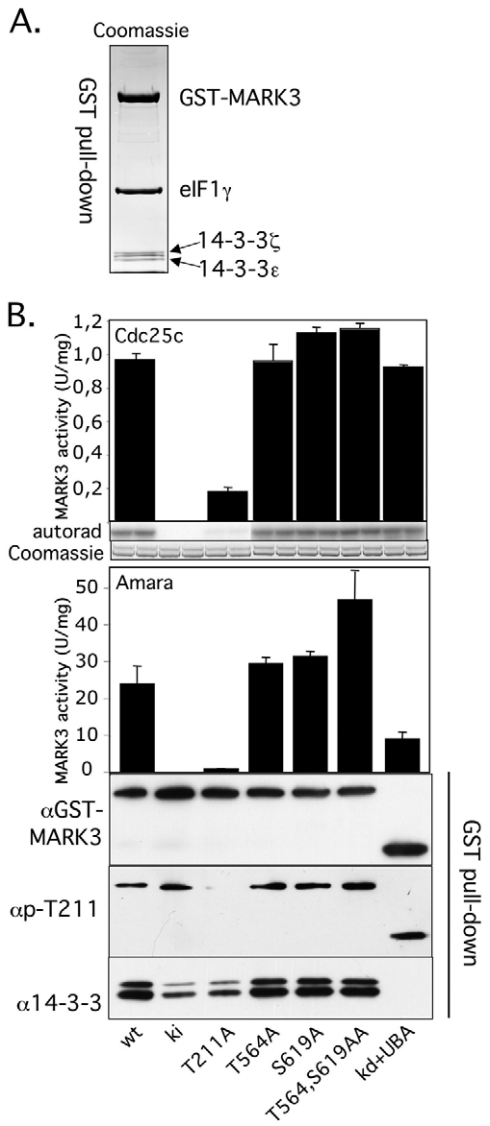



**Fig. 1.** (A) Identification of GST-MARK3 binding partners. Wild-type GST-MARK3 was expressed in HEK 293 cells, purified on glutathione-Sepharose, and subjected to SDS-PAGE. The gel was stained with colloidal Coomassie Blue, and protein bands were excised, washed and digested with trypsin. The identity of the interacting proteins was determined by mass spectrometry, as described previously (Al-Hakim et al., 2005). eIF1γ (eukaryotic elongation factor 1γ) is a non-specific binding-protein, observed to interact with most GST-fusion proteins expressed in HEK293 cells. (B) Analysis of various MARK mutants with regards to 14-3-3 binding and in vitro kinase activity. Wild-type (wt) or indicated mutant forms of GST-MARK3 (ki, kinase inactive D196A mutant; kd+UBA, kinase domain with ubiquitin-associated domain, residues 1-382), were expressed in HEK 293 cells, purified on glutathione-Sepharose and analysed by western blot with regards to total protein (αGST), T-loop phosphorylation (αp-T211) and 14-3-3 binding (α14-3-3). In vitro kinase activity was determined using the Cdc25c protein or AMARA peptide as substrate. The results are presented as the mean of a triplicate sample +s.d., and are representative of at least three experiments.

## Identification of novel MARK3 phosphorylation sites

The results shown in Figs 1 and 2 suggest that binding of MARK3 to 14-3-3 is mediated by phosphorylation of unknown residue(s). Therefore, we embarked on phosphopeptide mapping of MARK3, to identify novel sites of phosphorylation. Kinase-inactive MARK3, which interacts with 14-3-3 (Fig. 1B), was expressed in HEK 293 cells, digested with trypsin, and the resulting phosphopeptides analysed using LC/MS with precursor ion scanning (Fig. 3A). This revealed phosphorylation of MARK3 on the previously reported sites T211 (peak 5, Fig. 3A) and T564 (peak 20, Fig. 3A), as well as on 15 previously unreported residues. Twelve of the novel phosphorylation sites are located within the C-terminal spacer region, two are located in the N-terminal non-catalytic domain and one in the beginning of the kinase domain (Fig. 3B).

## Mutation of all MARK3 phosphorylation sites results in loss of 14-3-3 binding

To evaluate the role of individual MARK3 phosphorylation sites in binding to 14-3-3, single Ala point mutants of all the novel sites were generated, and analysed for their ability to bind 14-3-3. The three N-terminal sites (S42, S45 and T61), were mutated together. As demonstrated in Fig. 4A, all the phosphorylation-site mutants, apart from T211A, were active and phosphorylated at the T211 T-loop residue. Furthermore, all the mutants were capable of binding 14-3-3, at a level

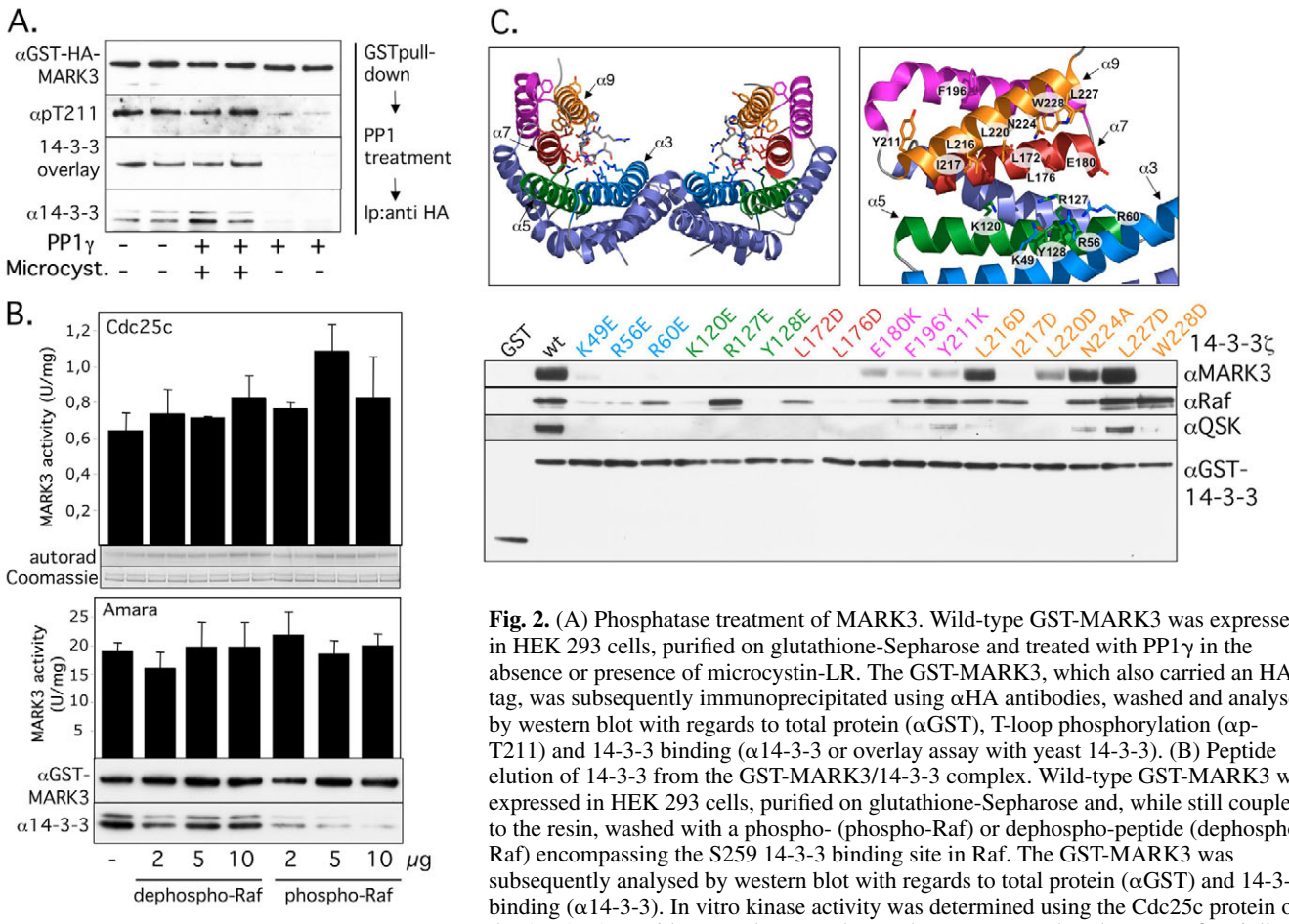


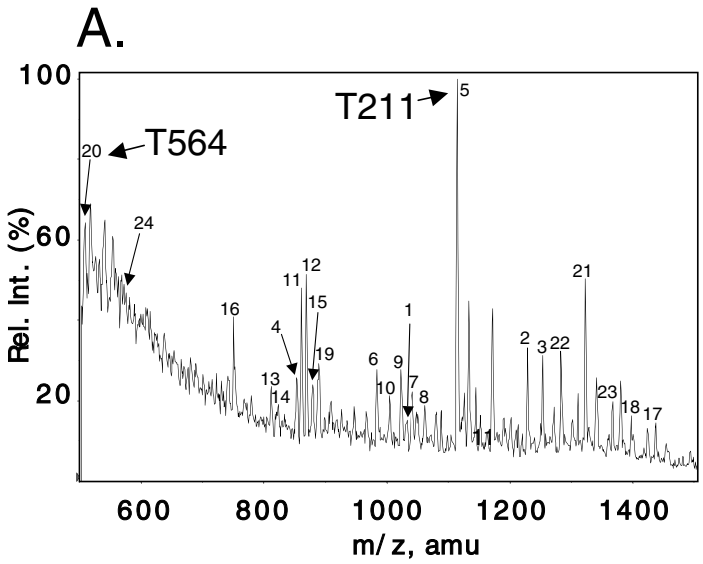

**Fig. 2.** (A) Phosphatase treatment of MARK3. Wild-type GST-MARK3 was expressed in HEK 293 cells, purified on glutathione-Sepharose and treated with PP1γ in the absence or presence of microcystin-LR. The GST-MARK3, which also carried an HA-tag, was subsequently immunoprecipitated using αHA antibodies, washed and analysed by western blot with regards to total protein (αGST), T-loop phosphorylation (αp-T211) and 14-3-3 binding (α14-3-3 or overlay assay with yeast 14-3-3). (B) Peptide elution of 14-3-3 from the GST-MARK3/14-3-3 complex. Wild-type GST-MARK3 was expressed in HEK 293 cells, purified on glutathione-Sepharose and, while still coupled to the resin, washed with a phospho- (phospho-Raf) or dephospho-peptide (dephospho-Raf) encompassing the S259 14-3-3 binding site in Raf. The GST-MARK3 was subsequently analysed by western blot with regards to total protein (αGST) and 14-3-3 binding (α14-3-3). In vitro kinase activity was determined using the Cdc25c protein or the AMARA peptide as a substrate. The results are presented as the mean of a triplicate sample + s.d., and are representative of at least three experiments. (C) Binding of various 14-3-3 mutants to endogenous MARK3, Raf and QSK. (Upper left panel) X-ray crystallographic structure of the 14-3-3ζ dimer in complex with a Raf peptide, as adapted from (Rittinger et al., 1999). The amphipathic cleft is formed by helix 3 (turquoise), helix 5 (green), helix 7 (red) and helix 9 (orange). (Upper right panel) Enlarged view of the amphipathic cleft with mutated residues indicated. (Bottom panel) Wild-type and indicated mutant forms of GST-14-3-3ζ, were expressed in HEK 293 cells, purified on glutathione-Sepharose and analysed by western blotting with regards to total protein (αGST), and binding to endogenous MARK3 (αMARK3), Raf (αRaf) and QSK (αQSK). Results shown are representative of two separate experiments. The E180K, F196Y and Y211K mutants studied previously are shown in pink (Benton et al., 2002).

similar to that of the wild-type protein. We also analysed two additional phosphorylation sites, T541 and S543, which were identified when analysing wild-type MARK3 (Fig. 3A), and therefore might represent autophosphorylation sites. A double mutant in which T541 and S543 were converted to Ala, was also active and capable of binding 14-3-3 (Fig. 4B).

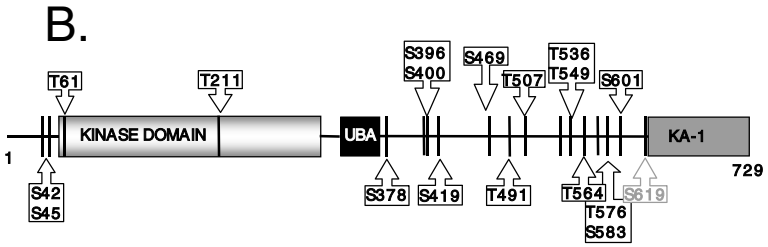
To confirm that the phosphorylation sites responsible for 14-3-3-binding are among the identified sites, we next created a mutant in which all phosphorylation sites were changed to Ala. This mutant contains 17 Ser/Thr to Ala mutations, and will hereafter be referred to as 17A-MARK3. As expected, when analysing the 17A-MARK3 mutant by LC/MS and precursor ion scanning, no phosphopeptides were detected (data not shown). Strikingly, this mutant was unable to interact with 14-3-3 (Fig. 5A). To investigate the effect that these mutations had on kinase activity, we generated a 17A-MARK3 version in which the T-loop T211 remained intact (termed 17A+T211-MARK3). Interestingly, despite possessing 16 mutations, this was found to be moderately more active than wild-type MARK3. The 17A+T211-MARK3 mutant was observed to interact with 14-3-3, albeit to a much lower extent than wild-type MARK3. This may be owing to the ability of MARK3 to autophosphorylate, because a kinase-inactive (D196A) 17A+T211-MARK3 mutant (ki 17A+T211-MARK3), that was phosphorylated on T211 failed to interact with 14-3-3 (Fig. 5B).

## Evidence that 14-3-3 binding serves to anchor MARK3 in the cytosol

We next investigated whether the subcellular localisation of MARK3 was influenced by its ability to bind 14-3-3. HEK 293 cells expressing either wild-type MARK3, T211A-MARK3, 17A+T211-MARK3 or 17A-MARK3, were fractionated into a cytosol- and a membrane fraction. As demonstrated in Fig. 6A, wild-type MARK3 and T211A-MARK3 were recovered both in the cytosolic- and in the membrane fraction, whereas the 14-3-3-binding defective mutants 17A+T211-MARK3 and 17A-MARK3 were exclusively recovered in the membrane fraction. There was no significant difference in the level of MARK3 T-loop T211 phosphorylation in the membrane- and cytosol fractions (Fig. 6A).




| peak no | detected phospho-peptides | phosphorylated residues |
|---|---|---|
| 1 | N**pS**IASCADEQPHIGNYR | S42 |
| 2 | CRN**pS**IASCADEQPHIGNYR | S42 |
| 3 | CRN**pS**IA**pS**CADEQPHIGNYR | S42/S45 |
| 4 | LLK**pT**IGK | T61 |
| 5 | LD**pT**FCGSPPYAAPELFQGK | T211 |
| 6 | SSELDASDSS**pS**SSNLSLAK | S378 |
| 7 | KSSELDASDSS**pS**SSNLSLAK | S378 |
| 8 | VRPSSDLNN**pS**TGQ**pS**PHHK | S396/S400 |
| 9 | VRPSSDLNNSTGQ**pS**PHHK | S400 |
| 10 | RY**pS**DHAGPAIPSVVAYPK | S419 |
| 11 | GIAPA**pS**PMLGNASNPNK | S469 |
| 12 | GIAPA**pS**PmLGNASNPNK | S469 |
| 13 | SS**pT**VPSSNTASGGMTR | T491 |
| 14 | SS**pT**VPSSNTASGGmTR | T491 |
| 15 | KSS**pT**VPSSNTASGGMTR | T491 |
| 16 | RN**pT**YVCSER | T507 |
| 17 | ENSTIPDQR**pT**PVASpTHpSISSAA**pT**PDR | T536/549, T541/S543* |
| 18 | ENSTIPDQRTPVASTHSISSAA**pT**PDR | T549 |
| 19 | TPVASTHSISSAA**pT**PDR | T549 |
| 20 | S**pT**FHGQPR | T564 |
| 21 | RTA**pT**YNGPPA**pS**PSLSHEATPLSQTR | T576/S583 |
| 22 | TATYNGPPA**pS**PSLSHEATPLSQTR | S583 |
| 23 | RTATYNGPPA**pS**PSLSHEATPLSQTR | S583 |
| 24 | SRG**pS**TNLFSK | S601 |




C.

| Res nr | hMARK1 | hMARK2 | hMARK3 | hMARK4 | xPAR-1 | cePAR-1 | dPAR-1 | yPAR-1 |
|---|---|---|---|---|---|---|---|---|
| 42 | √ | √ | √ | √ | √ | √ | √ | X |
| 45 | √ | √ | √ | √ | √ | X | X | X |
| 61 | √ | √ | √ | √ | √ | √ | √ | √ |
| 211 | √ | √ | √ | √ | √ | √ | √ | √ |
| 378 | √ | T | √ | X | √ | √ | √ | √ |
| 396 | √ | √ | √ | √ | √ | X | X | T |
| 400 | √ | X | √ | X | √ | X | √ | X |
| 419 | √ | √ | √ | √ | √ | X | √ | √ |
| 469 | √ | √ | √ | √ | √ | E | √ | √ |
| 491 | √ | X | √ | √ | √ | √ | X | X |
| 507 | √ | S | √ | √ | √ | S | X | X |
| 536 | X | X | √ | X | √ | X | X | X |
| 549 | √ | X | √ | S | √ | S | X | X |
| 564 | √ | √ | √ | √ | √ | √ | √ | √ |
| 576 | X | X | √ | X | √ | E | E | X |
| 583 | √ | √ | √ | √ | √ | X | X | X |
| 601 | √ | √ | √ | T | √ | X | X | X |
| 619 | √ | X | √ | T | √ | X | X | X |

**Fig. 3.** (A) Mapping of in vivo MARK3 phosphorylation sites. Kinase inactive (D196A) GST-MARK3 purified from HEK 293 cells on glutathione-Sepharose, was excised from a colloidal Coomassie-Blue-stained polyacrylamide gel, and digested with trypsin. One tenth of the digest was subjected to LC-MS with precursor ion scanning and the phosphorylated residues were identified by manual inspection of the acquired MSMS spectra (left panel) as described in Materials and Methods. The identified peaks are listed in the table, with phosphorylated residues in bold. Similar results were obtained in two subsequent experiments. In a further experiment, using wild type MARK3, the same sites were identified, except that T541 and S543 were assigned as the sites of phosphorylation in the ENSTIPDQRTPVASTHSISSAATPDR peptide (marked with an asterisk). (B) Location of phosphorylation sites in MARK3. Schematic view of MARK3, with each phosphopeptide represented by a box, in which phosphorylated residues have been listed. Phosphorylation sites are represented by vertical lines in the MARK3 structure. Phosphorylation of S619, reportedly phosphorylated in xPAR-1 (Kusakabe and Nishida, 2004), was not detected in our analysis but is included in the figure for reference. (C) Conservation of MARK3 phosphorylation sites. Human MARK3 amino acid sequence was aligned using Clustal W, with the indicated MARK homologues (human: hMARK1, hMARK2, hMARK4; *Xenopus*: xPAR-1; *C. elegans*: cePAR-1; *Drosophila*: dPAR-1; *S. cerevisiae* KIN1: yPAR-1. Conserved residues are indicated by a tick, no conservation by a cross. E, phosphorylation site is Gln; S, phosphorylation site is Ser rather than Thr; T, phosphorylation site is Thr rather than Ser.

Cellular localisation of green fluorescent protein (GFP) fusions of MARK3 proteins, expressed in HEK 293 cells, was examined by confocal fluorescence microscopy. In consistency with the results obtained in 6A, wild-type MARK3 was distributed throughout the cytoplasm, whereas the non-14-3-3-binding 17A-MARK3 mutant was strikingly localised at the plasma membrane (Fig. 6B). The T211A-MARK3 exhibited moderately increased plasma membrane localisation, in accordance with its reduced ability to bind 14-3-3 (Fig. 1B and Fig. 5A). 17A+T211-MARK3, which only bound 14-3-3 to a small extent (Fig. 5A), was primarily localised at the plasma membrane.

### Evidence that membrane localisation of MARK3 requires the KA-1 domain

We next investigated the mechanism whereby MARK3 is targeted to the membrane, and the possible role of the kinase-associated domain-1 (KA-1) domain. A C-terminal

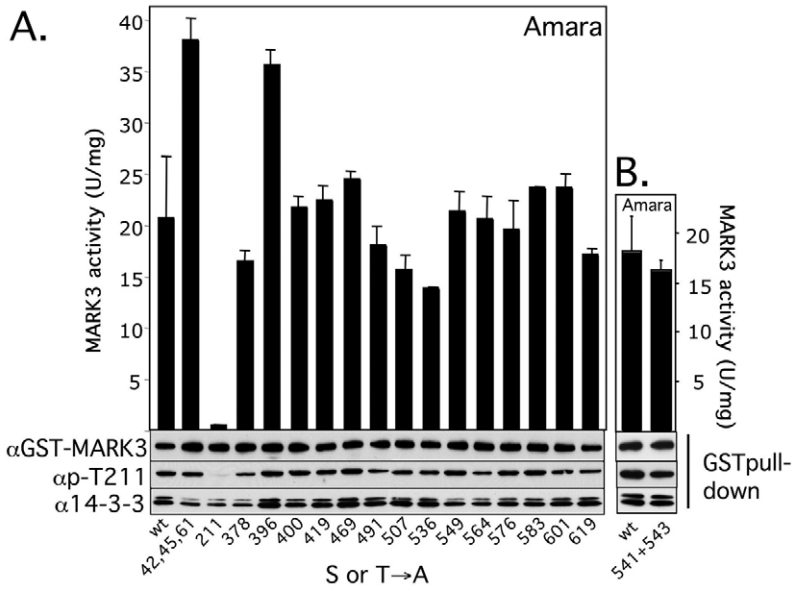



**Fig. 4.** Analysis of single MARK3 phosphorylation-site mutants. (A,B) Wild-type (wt) and indicated phosphorylation-site-mutant forms of GST-MARK3, were expressed in HEK 293 cells, purified on glutathione-Sepharose and analysed by western blot with regards to total MARK3 protein (αGST), T-loop phosphorylation (αp-T211) and 14-3-3 binding (α14-3-3). In vitro kinase activity was determined using the AMARA peptide substrate. S619 was not identified in our phosphopeptide mapping analysis but was mutated, because the equivalent site on xPAR-1 was reported to be phosphorylated by aPKC (Kusakabe and Nishida, 2004). The results are presented as the mean of a duplicate sample +s.d., and are representative of two experiments.

fragment of MARK3 (residues 327-729), encompassing the UBA domain, the spacer region and the KA-1 domain, was not able to bind 14-3-3 (Fig. 7A), and was primarily membrane localised (Fig. 7B). Removal of the KA-1 domain from this fragment (residues 327-630), did not induce 14-3-3 binding, but resulted in a loss of membrane localisation and increased cytosolic staining, suggesting that the KA-1 domain is required for membrane localisation of MARK3.

## Atypical PKC differently influences 14-3-3 binding and subcellular localisation of MARK isoforms

In a previous report, aPKC phosphorylation of xPAR-1b (equivalent to hMARK2) increased its association of 14-3-3 (Kusakabe and Nishida, 2004). Consistent with this observation, we demonstrate that overexpression of wild-type PKCζ, but not kinase-inactive PKCζ, increased several-fold the binding of 14-3-3 to MARK2 (Fig. 8A). This increase was prevented by mutation of the reported MARK2 PKCζ site (T508). By contrast, the ability of MARK3 to bind 14-3-3, was not affected by PKCζ overexpression (Fig. 8A). We next addressed whether this difference in PKCζ regulation of 14-3-3 binding, is reflected in a distinct subcellular localisation of MARK2 and MARK3 isoforms. We found that MARK2 was localised to the plasma membrane to a larger degree than MARK3, as judged by subcellular fractionation (compare Fig. 8B with Fig. 8D) and imaging of GFP-MARK2 expressed in

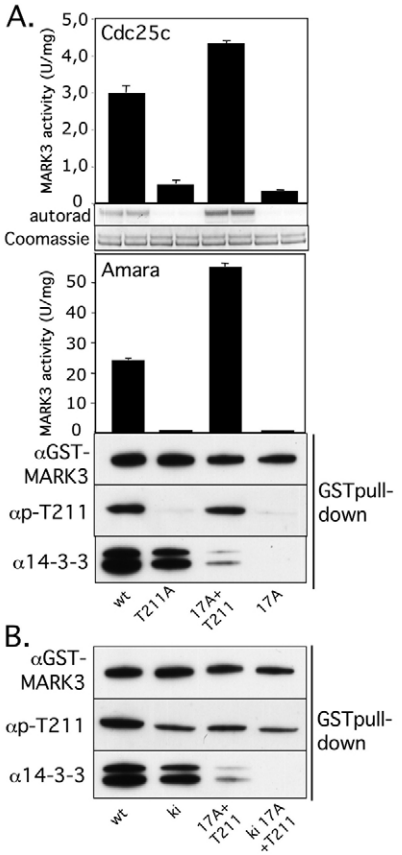



**Fig. 5.** Analysis of a total MARK3 phosphorylation-site mutant. (A,B) Wild-type (wt) and indicated mutant forms of GST-MARK3 (ki, kinase inactive D196A mutant), were expressed in HEK 293 cells, purified on glutathione-Sepharose and analysed by western blot with regards to total MARK3 protein (αGST), T-loop phosphorylation (αp-T211) and 14-3-3 binding (α14-3-3). In the 17A-MARK3 mutant, the 17 phosphorylation sites, indicated in Fig. 3 (S42, S45, T61, T211, S378, S396, S400, S419, S469, T491, T507, T536, T549, T564, T576, S583, S619), were converted to alanine residues. S619 was not identified in our phosphopeptide mapping analysis but was mutated, because the equivalent site on xPAR-1 was reported to be phosphorylated by aPKC (Kusakabe and Nishida, 2004). The 17A+T211-MARK3 mutant is identical to the 17A mutant, except that it has an intact T211 in the T-loop. In vitro kinase activity was determined using the protein substrate Cdc25c or the AMARA peptide substrate. The results are presented as the mean of a triplicate (AMARA) or duplicate (Cdc25c) sample + s.d., and are representative of at least three experiments.

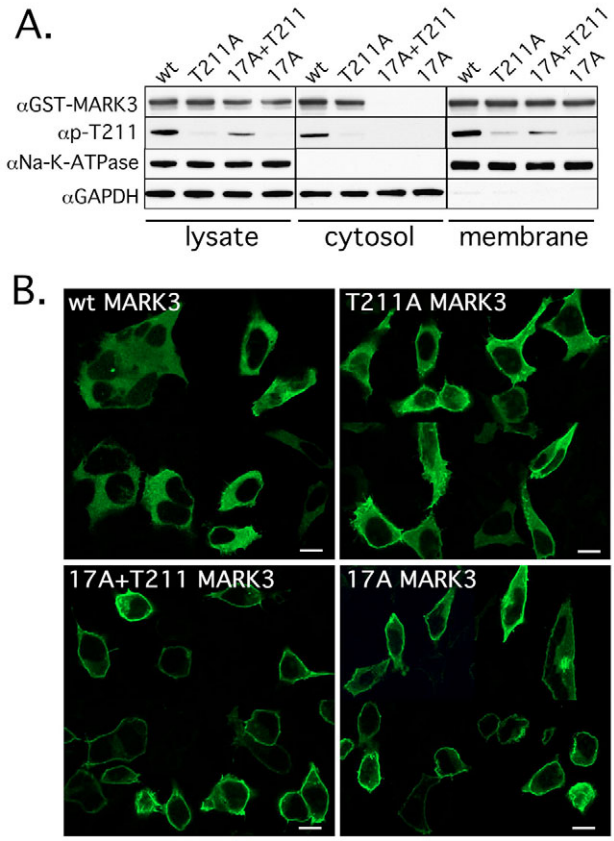



**Fig. 6.** Subcellular localisation of MARK3. (A) Wild-type (wt) or indicated mutant forms of GST-MARK3 (see Fig. 5) were expressed in HEK 293 cells, which were fractionated into a cytosolic and a membrane fraction. Subcellular fractions, including solubilised portions of the homogenates, were subsequently analysed by western blot with regards to MARK3 (αGST), MARK3 T-loop phosphorylation (αp-T211), the cytosolic marker GAPDH (αGAPDH) and the plasma membrane marker Na-K-ATPase (αNa-K-ATPase). The results are representative of three experiments. (B) Wild-type (wt) or indicated mutant forms of GFP-MARK3 (see Fig. 5) were expressed in HEK 293 cells, which were fixed in 3% paraformaldehyde 24 hours post transfection. The GFP-fluorescence was analysed by confocal fluorescence microscopy. The cells shown are representative of images obtained in two separate experiments. Bars, 10 μm.

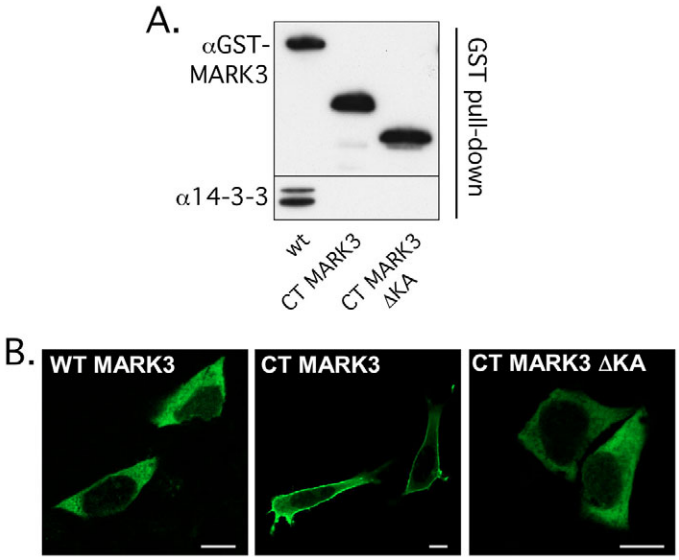



**Fig. 7.** (A) 14-3-3 binding to truncated MARK3. Wild-type (wt) or indicated truncated forms of GST-MARK3 (CT, residues 327-729; CTΔKA, residues 327-630) were expressed in HEK 293 cells, purified on glutathione-Sepharose and analysed by western blot with regards to total protein (αGST) and 14-3-3 binding (α14-3-3). (B) Subcellular localisation of truncated MARK3. Wild-type (wt) or indicated truncated forms of GFP-MARK3 were expressed in HEK 293 cells, which were fixed in 3% paraformaldehyde 24 hours post transfection. The GFP-fluorescence was analysed by confocal fluorescence microscopy. The cells shown are representative of images obtained in two separate experiments. Bars, 10 μm.

cells (compare Fig. 8C with Fig. 8E). Overexpression of wild-type PKCζ induced a marked increase in the cytoplasmic localisation of wild-type MARK2 (Fig. 8B and Fig. 8C, compare panels A and B), but not the T508A-MARK2 mutant (Fig. 8B and Fig. 8C, compare panels E and F). However, the localisation of wild-type or mutant forms of MARK3, was not significantly influenced by PKCζ co-expression (Fig. 8D,E), consistent with aPKC not controlling their association with 14-3-3.

MARK1 localisation was similar to that of MARK3, because MARK1 was recovered in both cytosolic and membrane fractions, and localised in both the cytoplasm and plasma membrane (supplementary material Fig. S1A and B, respectively). Mutation of the PKCζ sites in MARK1, resulted in a slight decrease of the amount of MARK1 that was recovered in the cytosolic fraction (supplementary material Fig. S1A). In subcellular fractionation studies, MARK4 was absent from the cytosolic fraction (supplementary material Fig. S1C) and, in confocal microscopy images, was observed to localise to filamentous structures (supplementary material Fig. S1D). We also noticed that PKCζ co-expression did not significantly influence the subcellular localisation of MARK1 or MARK4 (supplementary material Fig. S1).

## Discussion

An important conclusion of our work, is that phosphorylation of MARK3 is required for the binding of 14-3-3. A previous paper reported that interaction of 14-3-3 with dPAR-1 was disrupted by mutation of two 14-3-3 amino acid residues (F196 and Y211 in 14-3-3ζ) located outside the amphipathic cleft, whereas mutation of a residue inside the cleft (E180 in 14-3-3ζ) did not affect 14-3-3 binding (Benton et al., 2002). Based on these findings, it was concluded that dPAR-1 interacts with a hydrophobic area on the outside of the 14-3-3 molecule, rather than the Ser/Thr-binding pocket, and that interaction of dPAR-1 with 14-3-3 might be independent of phosphorylation (Benton et al., 2002). Our results, however, indicate that MARK3 does indeed dock within the Ser/Thr-binding pocket of 14-3-3, because mutation of E180 as well other residues within this region, markedly impaired 14-3-3 binding (Fig. 2C). We also noticed that mutation of F196 and Y211, located outside the phosphate-binding cleft, impaired 14-3-3 binding, suggesting that MARK3, in addition to interacting with the phosphate-binding pocket, makes contact with this region. Further evidence that phosphorylation of

MARK3 mediates 14-3-3 binding, results from the finding that phosphatase treatment ablated the ability of MARK3 to interact with 14-3-3 (Fig. 2A), and that the17A-MARK3 mutant no longer bound 14-3-3 (Fig. 5A).

In search for phosphorylated residues mediating 14-3-3 binding, we identified 15 novel in vivo phosphorylation sites in kinase-inactive MARK3. Interestingly, one of these sites, T61 is positioned in the beginning of the kinase domain, the structure of which was recently described for MARK2 (Panneerselvam et al., 2006). The corresponding MARK2 residue, T58, is situated close to the P-loop, in the N-terminal lobe of the kinase domain. However, T58 is positioned in a solvent-exposed area, some distance away from where the UBA domain interacts with the N-lobe. Phosphorylation of T58/T61 is therefore predicted not to interfere with catalysis or UBA domain binding. Three phosphorylation sites are conserved in all human MARK isoforms, as well as homologues from *Xenopus*, *Drosophila*, *C. elegans* and yeast, namely the T-loop T211, the aPKC site T564 and the N-terminal T61 (Fig. 3C). Strikingly, all identified MARK3 phosphorylation sites are conserved in xPAR-1a (equivalent to hMARK3) and, as summarised in Fig. 3C, many of the sites also displayed significant conservation among other MARK homologues. It will also be important to establish that these sites are phosphorylated on endogenously expressed MARK isoforms and to determine the relative stoichiometry of phosphorylation of these residues. It would also be crucial to establish the specific roles of individual phosphorylation sites in regulating the function of MARK3 and to identify the kinases which phosphorylate these residues. Interestingly, five

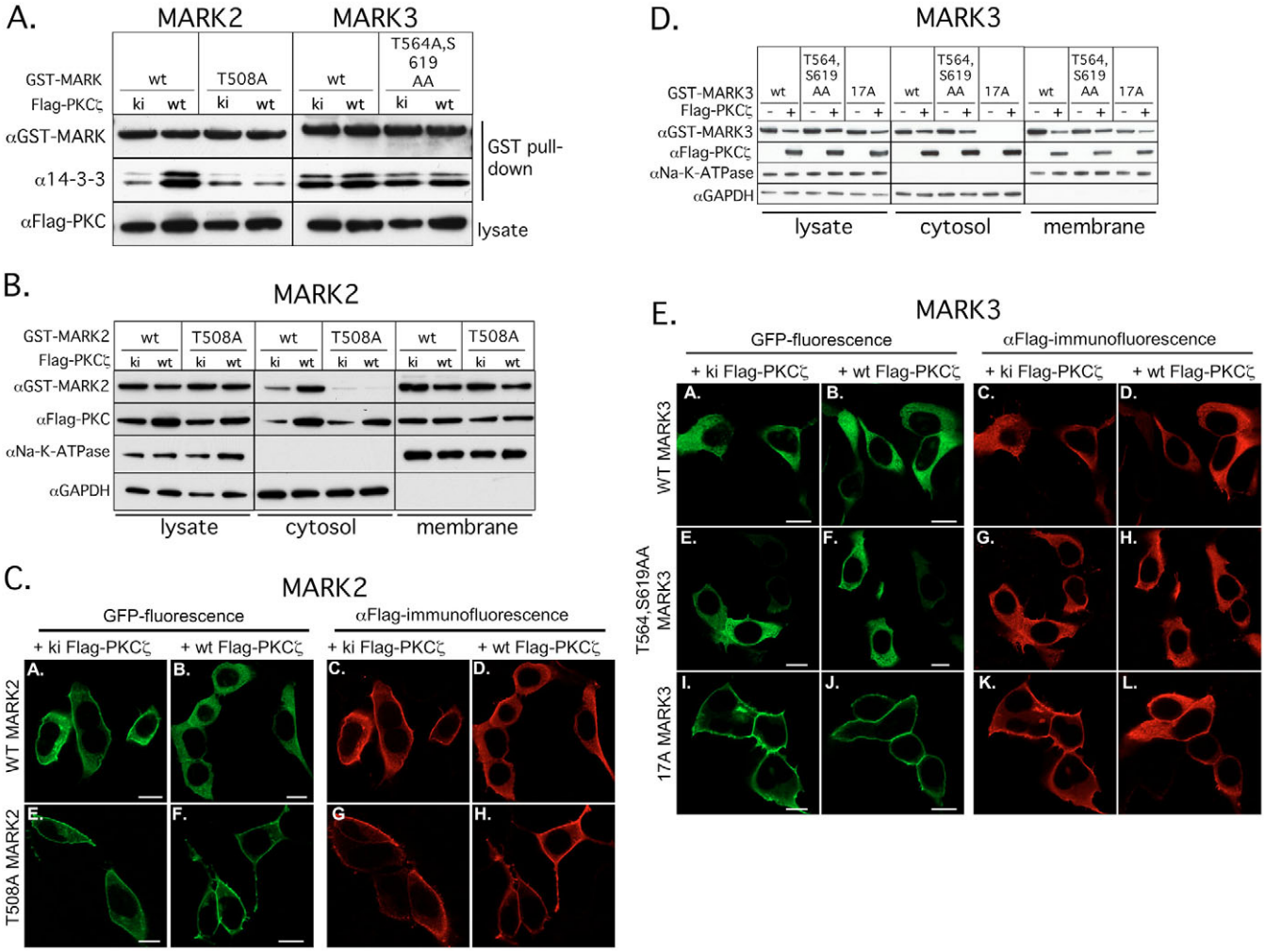



**Fig. 8.** (A) Binding of 14-3-3 to MARK2 and MARK3 after PKCζ co-expression. Wild-type (wt) or indicated mutant forms of GST-MARK isoforms were expressed in HEK 293 cells, in the absence or presence of kinase inactive (D394/A, ki) or wild-type (wt) Flag-PKCζ, purified on glutathione-Sepharose and analysed by western blot with regards to total protein (αGST) and 14-3-3 binding (α14-3-3). Expression of Flag-PKCζ was monitored by western blotting of the lysates (αFlag). (B-E) Subcellular localisation of MARK2 and MARK3 after PKCζ co-expression. (B,D) As in A, except cells were fractionated into a cytosolic and a membrane fraction. Subcellular fractions, including solubilised portions of the homogenates were analysed by western blot with regards to MARK isoforms (αGST), PKCζ (αFlag), the cytosolic marker GAPDH (αGAPDH) and the plasma membrane marker Na-K-ATPase (αNa-K-ATPase). (C,E) As in A, except that GFP fusions of MARK isoforms were employed and cells were fixed in 3% paraformaldehyde 24 hours post transfection. The cells were subsequently permeabilised and stained with anti-Flag and Alexa Fluor 594-labelled anti-mouse antibody. The fluorescence was analysed by confocal fluorescence microscopy. The cells shown are representative of images obtained in two separate experiments. Bars, 10 μm.

of the novel sites are followed by a proline residue, suggesting that a proline-directed kinase phosphorylates these sites. Glycogen synthase kinase 3 (GSK3) (Frame and Cohen, 2001), was recently suggested to phosphorylate MARK2, potentially at a T-loop residue (S312) located four residues C-terminal to the threonine phosphorylated by LKB1 (Kosuga et al., 2005). It would be interesting to investigate whether proline-directed kinases and GSK3 could phosphorylate MARK2 at sites other than S312, and whether these phosphorylations can be modulated by inhibitors and/or extracellular stimuli that affect proline-directed kinase/GSK3 activity.

Our results suggest that at least two phosphorylation sites have the capacity to, on their own, mediate 14-3-3 binding, because individual mutation of any of the identified phosphorylation sites did not ablate interaction (Fig. 4). Double mutants, in which the same individual mutations were made in a T211A or T564A background, were also still capable of binding 14-3-3 (O.G., unpublished). One or more auto-phosphorylation sites may contribute to 14-3-3 binding, because the interaction of 14-3-3 with kinase-inactive MARK3, as well as an inactive T-loop mutant, was slightly reduced (Fig. 5). Consistent with this, the kinase-inactive 17A+T211-MARK3 did not bind 14-3-3, whereas the active 17A+T211-MARK3 bound 14-3-3, albeit weakly (Fig. 5). The only candidate autophosphorylation sites that we were able to identify, comparing the phosphorylation of wild-type and kinase-inactive MARK3, were T541 and S543. Mutation of these sites did, however, not influence 14-3-3 binding. In an attempt to identify the specific phosphorylation sites capable of binding 14-3-3, we used the 17A-MARK3 mutant and reintroduced the phosphorylation sites one by one. However, apart from the T-loop T211, none of the other revertants bound 14-3-3 (data not shown). Truncated versions of MARK3 were also used to determine the regions of MARK3 that are required for 14-3-3 binding. However, fragments of MARK3 encompassing either the kinase domain and UBA domain (residues 1-382, Fig. 1B) or the UBA domain, the C-terminal spacer region and the KA-1 domain (residues 327-729, Fig. 7A), both failed to bind 14-3-3. It therefore appears that only the full-length MARK3 retains the ability to bind 14-3-3.

In xPAR-1b (equivalent to hMARK2), mutation of the two aPKC phosphorylation sites (T593 and S646) abolished interaction with 14-3-3ϵ (Kusakabe and Nishida, 2004). By contrast, mutation of the equivalent sites on MARK2 and MARK3 did not affect 14-3-3 binding (Fig. 8A). Our data is, however, consistent with aPKC regulating MARK2, because we show that binding of hMARK2 to 14-3-3, as well as its cytoplasmic localisation, is promoted by overexpression of PKCζ, and that this effect is prevented by mutation of the PKCζ phosphorylation site. In contrast to MARK2, MARK3 binding to 14-3-3, or its localisation, was unaffected by PKCζ overexpression, despite the high conservation in sequence surrounding the PKCζ phosphorylation site in MARK2 and MARK3 isoforms. The fact that MARK2 and MARK3, although similar in domain organisation and sequence, are regulated differently at the level of 14-3-3 binding and, hence, subcellular localisation, suggests that different MARK isoforms play distinct roles in the cell. Furthermore, MARK4, in contrast to other MARK isoforms, interacted with filamentous structures, the appearance of which resembled that of microtubules, which is consistent with a previous report, describing the subcellular distribution of MARK4 (Trinczek et al., 2004).

A previous report also indicated that MARK2 and MARK4, when overexpressed in HEK293 cells interacted more strongly with 14-3-3η than with 14-3-3ζ or 14-3-3β (Brajenovic et al., 2004). We identified 14-3-3ϵ and 14-3-3ζ associated with overexpressed MARK3, and failed to detect 14-3-3η or 14-3-3β (Fig. 1). Further studies would be required to establish whether different MARK isoforms possessed different affinities for 14-3-3 isoforms.

Our localisation studies demonstrated that, in the case of both MARK2 and MARK3, cytoplasmic localisation correlated with the ability of these enzymes to bind 14-3-3. The 17A-MARK3 mutant, which was unable to bind 14-3-3, was prominently present in the plasma membrane, in contrast to wild-type MARK3, which had a cytoplasmic localisation. Moreover, increased 14-3-3 binding to MARK2, as a result of PKCζ co-expression, induced a redistribution of MARK2 to the cytoplasm.

We have previously reported that binding of 14-3-3 to the T-loop of the AMPK-related kinases salt-inducible kinase (SIK) and QSK, resulted in a two- to threefold enhancement of kinase activity (Al-Hakim et al., 2005). However, MARK3 activity was not significantly affected by the dissociation of 14-3-3 (Fig. 2B). Furthermore, the 17A+T211-MARK3 mutant, which displayed markedly reduced 14-3-3 binding, was slightly more active than wild-type MARK3 (Fig. 5A). These observations indicate that 14-3-3 does not significantly influence MARK3 activity and that, apart from T211 in the T-loop, none of the other phosphorylation sites are required for activity. It was also reported for xPAR-1, that mutation of the aPKC sites, although abolishing 14-3-3 binding, did not affect activity (Kusakabe and Nishida, 2004).

As shown in Fig. 6, the non-14-3-3-binding forms of MARK3 are strikingly localised at the plasma membrane although MARK3 lacks any known membrane-targeting domain or motif. Our data indicate that the mechanism by which MARK3 is attached to the plasma membrane, requires the KA-1 domain (Fig. 7). Little is known about the role of this conserved C-terminal region of MARK isoforms, comprising ~100 amino acids. The KA-1 domain is found in MARK isoforms from all species, as well as in the maternal embryonic leucine zipper kinase (MELK) (Beullens et al., 2005), that is related to MARK isoforms but not activated by LKB1 (Lizcano et al., 2004). The KA-1 domain is not found in other proteins. In *Drosophila*, full-length PAR-1 and a splice variant lacking the KA-1 domain possessed similar subcellular localisation. However, the shorter variant rescued the phenotype of *Par-1* mutant *Drosophila* oocytes more efficiently than the long form (Huynh et al., 2001). Moreover, in the yeast MARK homologues KIN1 and KIN2, deletion of the KA-1 domain stimulated the function of the KIN1 and KIN2 enzymes in regulating the exocytic pathway (Elbert et al., 2005). The KA-1 domain in MELK reportedly plays an inhibitory role, because its deletion increased the in vitro activity of MELK towards protein and peptide substrates (Beullens et al., 2005). It was also observed that the KA-1 domain in KIN1, interacted with the kinase domain, inhibiting its function (Elbert et al., 2005). However, we were unable to detect an interaction of the C-terminal fragment of MARK3 (residues 327-729) with a fragment of MARK3 encompassing the kinase domain and

UBA domain (residues 1-382), when these were co-expressed in HEK 293 cells (O.G., unpublished). It would be interesting to investigate whether the KA-1 domain of KIN-1 and/or KIN2 regulates membrane binding of these enzymes. Others have suggested that protein-protein interaction mediates interaction of MARK isoforms with the plasma membrane, because association of MARK2 and MARK3 with membrane fractions was disrupted following a high-salt wash (Hurov et al., 2004). Identification of new MARK3-, and specifically KA-1-domain-interacting partners, might provide clues to how MARK3 membrane localisation and function is regulated.

## Materials and Methods

### Materials

Protein G–Sepharose, glutathione-Sepharose, $^{32}$Pγ-ATP and enhanced chemiluminescence reagent were purchased from Amersham Bioscience; protease-inhibitor-cocktail tablets, precast SDS polyacrylamide Bis-Tris gels and colloidal Coomassie Blue were from Invitrogen; phospho-cellulose P81 paper was from Whatman, and Microcystin-LR provided by Linda Lawton (Robert Gordon's University, Aberdeen). Peptides were synthesized by Graham Bloomberg at the University of Bristol. Human protein phosphatase-1 γ (PP1γ, GenBank accession number NM_002710) was expressed in *E.coli*, by the protein production team at the Division of Signal Transduction Therapy (DSTT), University of Dundee.

### Antibodies

The following antibodies were raised in sheep and affinity purified on the appropriate antigen: anti-phospho-T-loop MARK (residues 204-218 of human MARK3 phosphorylated at T211, TVGGKLDT(P)FCGSPPY), anti-QSK (residues 1349-1369 of human QSK, TDILLSYKHPEVSFSMEQAGV) and anti-GST (raised against the glutathione S-transferase protein). Polyclonal antibody recognising 14-3-3 isoforms was purchased from Santa Cruz Biotechnology (no. sc629), mouse monoclonal [4D11] anti-His antibody, monoclonal anti-GAPDH antibody and monoclonal anti-Na-K ATPase antibody were from Abcam (no. ab5000-100, no. 9484 and no. ab7671, respectively), monoclonal antibodies recognising the HA-epitope tag was from Roche (no. 1666606), anti-Raf1 antibody from Upstate (no. 05-739), and anti-Flag antibody from Sigma (no. F3165). Secondary antibodies coupled to horseradish peroxidase (HRP) were obtained from Pierce, and Alexa Fluor 594-labelled anti-mouse antibody used for localisation studies was from Molecular Probes (no. A21201).

### General methods

Tissue culture, transfection, western blotting, restriction enzyme digests, DNA ligations, and other recombinant DNA procedures were performed using standard protocols. The generation of cDNA clones for human MARK1 (NCBI Acc. NM_018650), MARK2 (NCBI Acc. NM_004954), MARK3 (NCBI Acc. NM_002376), MARK4 (NCBI Acc. AK075272), 14-3-3ζ (Genbank Acc. NM_145690) and PKCζ (NCBI Acc. M94632), has been described previously (Al-Hakim et al., 2005; Balendran et al., 2000; Lizcano et al., 2004). All mutagenesis was carried out using the Quick-Change site-directed mutagenesis method (Stratagene). DNA constructs used for transfection were purified from *E. coli* DH5α using Qiagen plasmid High-speed Maxi kit according to the manufacturer's protocol. All DNA constructs were verified by DNA sequencing, which was performed by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland, UK, using DYEnamic ET terminator chemistry (Amersham Biosciences) on Applied Biosystems automated DNA sequencers.

### Buffers

Lysis Buffer contained: 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (w/v) NP-40, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and complete proteinase inhibitor cocktail (one tablet/50 ml). Buffer A contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA and 1 mM DTT. TBS-Tween buffer contained: 50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.2% (v/v) Tween-20.

### Expression and purification of Cdc25c in *E. coli*

The pGEX expression construct encoding human full-length Cdc25c, kindly provided by Helen Piwnica-Worms, was transformed into *E.coli* BL21 cells. One-litre cultures were grown at 37°C in Luria broth containing 100 μg/ml ampicillin until the absorbance at 600 nm was 0.8. Induction of protein expression was carried out by adding 500 μM isopropyl-β-D-galactoside and the cells were cultured for a further 3 hours at 30°C. Cells were isolated by centrifugation, resuspended in 25 ml of ice-cold lysis buffer and lysed in one round of freeze-thawing, followed by sonication to fragment DNA. The lysates were centrifuged at 4°C for 30 minutes at 30,000 ***g***, and the recombinant proteins were affinity purified on glutathione-Sepharose and eluted in buffer A containing 20 mM glutathione and 0.27 M sucrose.

### Expression and purification of GST-MARK isoforms and GST-14-3-3ζ in HEK 293 cells

Five 10-cm diameter dishes of HEK 293 cells were cultured and each dish was transfected with 10 μg of the pEBG-2T construct encoding wild-type or indicated mutant forms of human MARK isoforms and human 14-3-3ζ, using the polyethylenimine method (Durocher et al., 2002). Cells were cultured for a further 36 hours and subsequently lysed in 0.5 ml of ice-cold lysis buffer per dish. The lysates were pooled and centrifuged at 4°C for 10 minutes at 26,000 ***g***, and the GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose and eluted in buffer A containing 20 mM glutathione and 0.27 M sucrose.

### Western blotting

Total cell lysate (5-50 μg) or purified proteins (50 ng-2 μg) were heated at 70°C for 5 minutes in SDS sample buffer, and subjected to polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membrane. Membranes were blocked for 30 minutes in TBS-Tween buffer containing 10% (w/v) skimmed milk. The membranes were then probed with 0.5-1 μg/ml of indicated antibodies in TBS-Tween, 5% (w/v) skimmed milk for 16 hours at 4°C. Detection was performed using HRP-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

### Measurement of MARK3 kinase activity

The activity of MARK3 was quantified by measurement of phosphorylation of the AMARA (AMARAASAAALRRR) peptide substrate (Lizcano et al., 2004) or the previously identified full-length protein substrate Cdc25c (Peng et al., 1998). For the kinase activity assay using AMARA peptide as a substrate, 50-100 ng of purified MARK3 was incubated in a 50-μl mixture containing 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM [γ-$^{32}$P]-ATP (300 cpm/pmol) and 200 μM AMARA peptide for 30 minutes at 30°C. Incorporation of $^{32}$P-phosphate into the peptide substrate was determined by applying 40 μl of the reaction mixture onto P81 phospho-cellulose paper, followed by washing of the papers in 50 mM phosphoric acid and scintillation counting. One unit (U) of activity was defined as that which catalysed the incorporation of 1 nmol of $^{32}$P into the substrate. For the Cdc25c protein substrate assay, 100-150 ng MARK3 was incubated for 30 minutes at 30°C in a volume of 30 μl containing 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM [γ-$^{32}$P]-ATP (300 cpm/pmol) and 1 μg of GST-Cdc25c. Following polyacrylamide gel electrophoresis, Coomassie-staining and autoradiography, the full-length GST-Cdc25c bands were excised and the incorporation of $^{32}$P was determined by Cerenkov counting.

### 14-3-3 overlay assay

GST-MARK3 (500 ng) was subjected to polyacrylamide gel electrophoresis and electrotransfer to nitrocellulose membrane. Overlay assays were undertaken using a previously described method (Moorhead et al., 1996). Briefly, membranes were blocked for 1 hour in TBS-Tween buffer containing 5% (w/v) skimmed milk and 0.5 M NaCl. The membranes were then incubated with 5 μg/ml total His-BMH1 and His-BMH2 (yeast 14-3-3 isoforms, expressed in *E. coli*), in TBS-Tween containing 1 mg/ml BSA and 0.5 M NaCl for 16 hours at room temperature. The membranes were washed six times for 5 minutes with TBS-Tween containing 0.5 M NaCl and probed with a 1:5000 dilution of anti-His antibody in TBS- Tween containing 5% (w/v) skimmed milk and 0.5 M NaCl, for 1 hour at room temperature. Detection was performed using HPR-conjugated secondary antibodies and the enhanced chemiluminescence reagent.

### Protein phosphatase treatment of MARK3

GST-HA-MARK3 purified from HEK 293 cells (0.5-1 μg) was incubated in a 30-μl mixture of 50 mM Tris-HCl pH 7.5, 1 mM DTT, 0.1 mM EGTA, 1 mM $MnCl_2$ and 2.5 mU PP1γ, with or without 0.5 μg Microcystin-LR, for 30 minutes at 30°C. The reaction was terminated by the addition of 0.5 μg microcystin-LR, and the GST-HA-MARK3 immunoprecipitated using 1 μg of anti-HA antibody. The washed immunoprecipitates were subsequently analysed by Western blotting and 14-3-3 overlay assay as described above.

### Dissociation of 14-3-3 isoforms from MARK3 using a Raf peptide

GST-MARK3 was expressed in HEK 293 cells, absorbed onto glutathione-Sepharose and washed with Buffer A, as described above. Aliquots of 20 μl of glutathione-Sepharose, still conjugated to GST-MARK3, were mixed with 150 μl of Buffer A containing either no peptide or increasing amounts of a Raf phospho- or dephosphopeptide (residues 251-266, LSQRQRST(p)STPNVHMV). After incubation for 20 min at 4°C on a vibrating platform, the beads were pelleted, the supernatant removed, and the incubation repeated. The Sepharose was subsequently washed twice with 1 ml of Buffer A containing 0.27 M sucrose and the GST-

MARK3 eluted in 50 μl of Buffer A containing 20 mM glutathione and 0.27 M sucrose. MARK3 activity measurements and western blot analysis was performed on the eluted protein as described above.

### Mapping in vivo phosphorylation sites in GST-MARK3

Wild-type or kinase-inactive (D196A) GST-MARK3 (10 μg) purified from HEK 293 cells was incubated in 50 mM iodoacetamide for 30 minutes at room temperature to alkylate Cys residues, and then subjected to polyacrylamide gel electrophoresis. The gel was stained with colloidal Coomassie Blue, and the GST-MARK3 band was excised, washed and digested with trypsin for 16 hours. To determine the phosphorylated residues, the digests were reconstituted in 0.1 ml 1% (v/v) formic acid in water and analysed by LC-MS on a 4000 Q-TRAP system after prior enrichment using Phos-Select immobilised metal affinity chromatography (IMAC) resin, as described previously (Lochhead et al., 2005), or by LC-MS without prior enrichment using precursor ion scanning (Williamson et al., 2006). All the MS/MS spectra were searched against local databases using the Mascot search engine (MatrixScience) and sites of phosphorylation were manually assigned from individual MS/MS spectra viewed using Bioanalyst software (MDS-Sciex).

### Subcellular fractionation

HEK 293 cells, transfected with indicated pEBG-2T (GST) MARK constructs and/or pCMV Flag-PKCζ constructs, were scraped in lysis buffer without NP40 and homogenised by passing ten times through a chamber containing a ball bearing, in which the space between the chamber wall and the ball bearing was 0.014 mm. To generate a total lysate, at this stage, a portion of the homogenate was taken off, supplemented with 1% (v/v) NP40, left on ice to solubilise for 30 minutes and centrifuged at 16,000 ***g*** for 10 minutes. The remainder of the homogenate was centrifuged at 1500 ***g*** for 5 minutes, to pellet nuclei and unbroken cells, and the resulting supernatant was centrifuged at 100,000 ***g*** for 1 hour. The supernatant was taken as the cytosol fraction, and the pellet, referred to as the membrane fraction, was resuspended and homogenised in 0.5 ml of lysis buffer without NP40. All fractions were supplemented with 1% (v/v) NP40, left on ice to solubilise for 30 minutes, and centrifuged at 16,000 ***g*** for 10 minutes, to pellet insoluble material. Equal amounts of total protein (5-10 μg) from each fraction, were loaded on polyacrylamide gels and analysed by western blot.

### Localisation of GFP fusion proteins

HEK 293 cells were cultured to 50% confluence on glass cover slips (no. 1.5) in 6-well plates, and transfected with 0.5-1.0 μg of pEGFP constructs encoding wild-type or indicated MARK mutants, and/or pCMV constructs encoding wild-type or kinase inactive Flag-PKCζ, using the polyethylenimine method (Durocher et al., 2002). Twenty-four hours post-transfection cells were washed with PBS, and fixed in 3% (v/v) paraformaldehyde in PBS (Oxoid Limited, no. BR0014G) for 10 minutes. In PKC co-expression experiments, the cells were permeabilised for 5 minutes with 1% (w/v) Triton X-100 in PBS, and subsequently stained with mouse anti-Flag antibodies and 2 μg/ml of Alexa Fluor-594-labelled anti-mouse antibody (Molecular Probes). Optical sections of 0.5 μm were taken with a Zeiss LSM 510 META confocal microscope, with an alpha Plan-Fluar ×100 objective (NA 1.45).

We thank Carol MacKintosh for helpful advice, and reagents, Helen Piwnica-Worms for providing the Cdc25c construct, Fabrizio Villa for help with preparing a high-resolution image of the 14-3-3ζ structure, Mark Peggie for cloning and generation of expression constructs for 14-3-3ζ, Agnieszka Kieloch for tissue culture, the Sequencing Service (School of Life Sciences, University of Dundee, Scotland) for DNA sequencing, the Post Genomics and Molecular Interactions Centre for Mass Spectrometry facilities (School of Life Sciences, University of Dundee, Scotland) and the protein production and antibody purification teams (Division of Signal Transduction Therapy (DSTT), University of Dundee), co-ordinated by Hilary McLauchlan and James Hastie, for purification of antibodies. O.G. is supported by a Wenner-Gren Foundation fellowship. We thank the Association for International Cancer Research, Diabetes UK, the Medical Research Council, the Moffat Charitable Trust and the pharmaceutical companies supporting the Division of Signal Transduction Therapy Unit (AstraZeneca, Boehringer-Ingelheim, GlaxoSmithKline, Merck & Co. Inc, Merck KgaA and Pfizer) for financial support. We also acknowledge the BBSRC and EPSCR (award BB/C511613/1) for funding the mass spectrometer system used in this study.

## References

**Al-Hakim, A. K., Goransson, O., Deak, M., Toth, R., Campbell, D. G., Morrice, N. A., Prescott, A. R. and Alessi, D. R.** (2005). 14-3-3 cooperates with LKB1 to regulate the activity and localization of QSK and SIK. *J. Cell Sci.* **118**, 5661-5673.

**Alessi, D. R., Sakamoto, K. and Bayascas, J. R.** (2006). LKB1-dependent signaling pathways. *Annu. Rev. Biochem.* **75**, 137-163.

**Balendran, A., Biondi, R. M., Cheung, P. C., Casamayor, A., Deak, M. and Alessi, D. R.** (2000). A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1. *J. Biol. Chem.* **275**, 20806-20813.

**Beghini, A., Magnani, I., Roversi, G., Piepoli, T., Di Terlizzi, S., Moroni, R. F., Pollo, B., Fuhrman Conti, A. M., Cowell, J. K., Finocchiaro, G. et al.** (2003). The neural progenitor-restricted isoform of the MARK4 gene in 19q13.2 is upregulated in human gliomas and overexpressed in a subset of glioblastoma cell lines. *Oncogene* **22**, 2581-2591.

**Benton, R. and St Johnston, D.** (2003). Drosophila PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. *Cell* **115**, 691-704.

**Benton, R., Palacios, I. M. and St Johnston, D.** (2002). Drosophila 14-3-3/PAR-5 is an essential mediator of PAR-1 function in axis formation. *Dev. Cell* **3**, 659-671.

**Beullens, M., Vancauwenbergh, S., Morrice, N., Derua, R., Ceulemans, H., Waelkens, E. and Bollen, M.** (2005). Substrate specificity and activity regulation of protein kinase MELK. *J. Biol. Chem.* **280**, 40003-40011.

**Biernat, J., Wu, Y. Z., Timm, T., Zheng-Fischhofer, Q., Mandelkow, E., Meijer, L. and Mandelkow, E. M.** (2002). Protein kinase MARK/PAR-1 is required for neurite outgrowth and establishment of neuronal polarity. *Mol. Biol. Cell* **13**, 4013-4028.

**Bohm, H., Brinkmann, V., Drab, M., Henske, A. and Kurzchalia, T. V.** (1997). Mammalian homologues of C. elegans PAR-1 are asymmetrically localized in epithelial cells and may influence their polarity. *Curr. Biol.* **7**, 603-606.

**Brajenovic, M., Joberty, G., Kuster, B., Bouwmeester, T. and Drewes, G.** (2004). Comprehensive proteomic analysis of human Par protein complexes reveals an interconnected protein network. *J. Biol. Chem.* **279**, 12804-12811.

**Brown, A. J., Hutchings, C., Burke, J. F. and Mayne, L. V.** (1999). Application of a rapid method (targeted display) for the identification of differentially expressed mRNAs following NGF-induced neuronal differentiation in PC12 cells. *Mol. Cell Neurosci.* **13**, 119-130.

**Chang, H. C. and Rubin, G. M.** (1997). 14-3-3 epsilon positively regulates Ras-mediated signaling in Drosophila. *Genes Dev.* **11**, 1132-1139.

**Cohen, D., Brennwald, P. J., Rodriguez-Boulan, E. and Musch, A.** (2004). Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton. *J. Cell Biol.* **164**, 717-727.

**Drewes, G.** (2004). MARKing tau for tangles and toxicity. *Trends Biochem. Sci.* **29**, 548-555.

**Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E. M. and Mandelkow, E.** (1997). MARK, a novel family of protein kinases that phosphorylate microtubule-associated proteins and trigger microtubule disruption. *Cell* **89**, 297-308.

**Durocher, Y., Perret, S. and Kamen, A.** (2002). High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. *Nucleic Acids Res.* **30**, E9.

**Elbert, M., Rossi, G. and Brennwald, P.** (2005). The yeast par-1 homologs kin1 and kin2 show genetic and physical interactions with components of the exocytic machinery. *Mol. Biol. Cell* **16**, 532-549.

**Frame, S. and Cohen, P.** (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J.* **359**, 1-16.

**Guo, S. and Kemphues, K. J.** (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611-620.

**Hashiguchi, M., Sobue, K. and Paudel, H. K.** (2000). 14-3-3zeta is an effector of tau protein phosphorylation. *J. Biol. Chem.* **275**, 25247-25254.

**Hurov, J. B., Watkins, J. L. and Piwnica-Worms, H.** (2004). Atypical PKC phosphorylates PAR-1 kinases to regulate localization and activity. *Curr. Biol.* **14**, 736-741.

**Huynh, J. R., Shulman, J. M., Benton, R. and St Johnston, D.** (2001). PAR-1 is required for the maintenance of oocyte fate in Drosophila. *Development* **128**, 1201-1209.

**Jin, J., Smith, F. D., Stark, C., Wells, C. D., Fawcett, J. P., Kulkarni, S., Metalnikov, P., O'Donnell, P., Taylor, P., Taylor, L. et al.** (2004). Proteomic, functional, and domain-based analysis of in vivo 14-3-3 binding proteins involved in cytoskeletal regulation and cellular organization. *Curr. Biol.* **14**, 1436-1450.

**Kato, T., Satoh, S., Okabe, H., Kitahara, O., Ono, K., Kihara, C., Tanaka, T., Tsunoda, T., Yamaoka, Y., Nakamura, Y. et al.** (2001). Isolation of a novel human gene, MARKL1, homologous to MARK3 and its involvement in hepatocellular carcinogenesis. *Neoplasia* **3**, 4-9.

**Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. S.** (1988). Identification of genes required for cytoplasmic localization in early C. elegans embryos. *Cell* **52**, 311-320.

**Kosuga, S., Tashiro, E., Kajioka, T., Ueki, M., Shimizu, Y. and Imoto, M.** (2005). GSK-3beta directly phosphorylates and activates MARK2/PAR-1. *J. Biol. Chem.* **280**, 42715-42722.

**Kusakabe, M. and Nishida, E.** (2004). The polarity-inducing kinase Par-1 controls Xenopus gastrulation in cooperation with 14-3-3 and aPKC. *EMBO J.* **23**, 4190-4201.

**La Carbona, S., Allix, C., Philippe, M. and Le Goff, X.** (2004). The protein kinase kin1 is required for cellular symmetry in fission yeast. *Biol. Cell* **96**, 169-179.

**Layfield, R., Fergusson, J., Aitken, A., Lowe, J., Landon, M. and Mayer, R. J.** (1996). Neurofibrillary tangles of Alzheimer's disease brains contain 14-3-3 proteins. *Neurosci. Lett.* **209**, 57-60.

**Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H. and Liddington, R.** (1995). Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* **376**, 191-194.

**Lizcano, J. M., Goransson, O., Toth, R., Deak, M., Morrice, N. A., Boudeau, J., Hawley, S. A., Udd, L., Makela, T. P., Hardie, D. G. et al.** (2004). LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *EMBO J.* **23**, 833-843.

**Lochhead, P. A., Sibbet, G., Morrice, N. and Cleghon, V.** (2005). Activation-loop autophosphorylation is mediated by a novel transitional intermediate form of DYRKs. *Cell* **121**, 925-936.

**Mackintosh, C.** (2004). Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem. J.* **381**, 329-342.

**Moorhead, G., Douglas, P., Morrice, N., Scarabel, M., Aitken, A. and MacKintosh, C.** (1996). Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. *Curr. Biol.* **6**, 1104-1113.

**Muller, J., Ory, S., Copeland, T., Piwnica-Worms, H. and Morrison, D. K.** (2001). C-TAK1 regulates Ras signaling by phosphorylating the MAPK scaffold, KSR1. *Mol. Cell* **8**, 983-993.

**Muller, J., Ritt, D. A., Copeland, T. D. and Morrison, D. K.** (2003). Functional analysis of C-TAK1 substrate binding and identification of PKP2 as a new C-TAK1 substrate. *EMBO J.* **22**, 4431-4442.

**Nishimura, I., Yang, Y. and Lu, B.** (2004). PAR-1 kinase plays an initiator role in a temporally ordered phosphorylation process that confers tau toxicity in Drosophila. *Cell* **116**, 671-682.

**Panneerselvam, S., Marx, A., Mandelkow, E. M. and Mandelkow, E.** (2006). Structure of the catalytic and ubiquitin-associated domains of the protein kinase MARK/Par-1. *Structure* **14**, 173-183.

**Pellettieri, J. and Seydoux, G.** (2002). Anterior-posterior polarity in C. elegans and Drosophila – PARallels and differences. *Science* **298**, 1946-1950.

**Peng, C. Y., Graves, P. R., Ogg, S., Thoma, R. S., Byrnes, M. J., 3rd, Wu, Z., Stephenson, M. T. and Piwnica-Worms, H.** (1998). C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell Growth Differ.* **9**, 197-208.

**Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H. and Liddington, R. C.** (1998). 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. *J. Biol. Chem.* **273**, 16305-16310.

**Rittinger, K., Budman, J., Xu, J., Volinia, S., Cantley, L. C., Smerdon, S. J., Gamblin, S. J. and Yaffe, M. B.** (1999). Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol. Cell* **4**, 153-166.

**Schneider, A., Laage, R., von Ahsen, O., Fischer, A., Rossner, M., Scheek, S., Grunewald, S., Kuner, R., Weber, D., Kruger, C. et al.** (2004). Identification of regulated genes during permanent focal cerebral ischaemia: characterization of the protein kinase 9b5/MARKL1/MARK4. *J. Neurochem.* **88**, 1114-1126.

**Sebastian, B., Kakizuka, A. and Hunter, T.** (1993). Cdc25M2 activation of cyclin-dependent kinases by dephosphorylation of threonine-14 and tyrosine-15. *Proc. Natl. Acad. Sci. USA* **90**, 3521-3524.

**Shulman, J. M., Benton, R. and St Johnston, D.** (2000). The Drosophila homolog of C. elegans PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. *Cell* **101**, 377-388.

**Tassan, J. P. and Le Goff, X.** (2004). An overview of the KIN1/PAR-1/MARK kinase family. *Biol. Cell* **96**, 193-199.

**Timm, T., Li, X. Y., Biernat, J., Jiao, J., Mandelkow, E., Vandekerckhove, J. and Mandelkow, E. M.** (2003). MARKK, a Ste20-like kinase, activates the polarity-inducing kinase MARK/PAR-1. *EMBO J.* **22**, 5090-5101.

**Trinczek, B., Brajenovic, M., Ebneth, A. and Drewes, G.** (2004). MARK4 is a novel microtubule-associated proteins/microtubule affinity-regulating kinase that binds to the cellular microtubule network and to centrosomes. *J. Biol. Chem.* **279**, 5915-5923.

**Wang, H., Zhang, L., Liddington, R. and Fu, H.** (1998). Mutations in the hydrophobic surface of an amphipathic groove of 14-3-3zeta disrupt its interaction with Raf-1 kinase. *J. Biol. Chem.* **273**, 16297-16304.

**Williamson, B. L., Marchese, J. and Morrice, N. A.** (2006). Automated identification and quantification of protein phosphorylation sites by LC/MS on a hybrid triple quadrupole linear ion trap mass spectrometer. *Mol. Cell Proteomics* **5**, 337-346.

**Zhang, L., Wang, H., Liu, D., Liddington, R. and Fu, H.** (1997). Raf-1 kinase and exoenzyme S interact with 14-3-3zeta through a common site involving lysine 49. *J. Biol. Chem.* **272**, 13717-13724.

**Zhang, L., Wang, H., Masters, S. C., Wang, B., Barbieri, J. T. and Fu, H.** (1999). Residues of 14-3-3 zeta required for activation of exoenzyme S of Pseudomonas aeruginosa. *Biochemistry* **38**, 12159-12164.

**Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H. and Tonks, N. K.** (1997). Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. *J. Biol. Chem.* **272**, 27281-27287.