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Substrate preference and phosphatidylinositol monophosphate inhibition of the catalytic domain of the Per-Arnt-Sim domain kinase PASKIN

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The Per-Arnt-Sim (PAS) domain serine/threonine kinase PASKIN, or PAS kinase, links energy flux and protein synthesis in yeast, regulates glycogen synthesis and protein translation in mammals, and might be involved in insulin regulation in the pancreas. According to the current model, binding of a putative ligand to the PAS domain disinhibits the kinase domain, leading to PASKIN autophosphorylation and increased kinase activity. To date, only synthetic but no endogenous PASKIN ligands have been reported. In the present study, we identified a number of novel PASKIN kinase targets, including ribosomal protein S6. Together with our previous identification of eukaryotic elongation factor 1A1, this suggests a role for PASKIN in the regulation of mammalian protein translation. When searching for endogenous PASKIN ligands, we found that various phospholipids can bind PASKIN and stimulate its autophosphorylation. Interestingly, the strongest binding and autophosphorylation was achieved with monophosphorylated phosphatidylinositols. However, stimulated PASKIN autophosphorylation did not correlate with ribosomal protein S6 and eukaryotic elongation factor 1A1 target phosphorylation. Although autophosphorylation was enhanced by monophosphorylated phosphatidylinositols, di- and tri-phosphorylated phosphatidylinositols inhibited autophosphorylation. By contrast, target phosphorylation was always inhibited, with the highest efficiency for di- and tri-phosphorylated phosphatidylinositols. Because phosphatidylinositol monophosphates were found to interact with the kinase rather than with the PAS domain, these data suggest a multiligand regulation of PASKIN activity, including a still unknown PAS domain binding/activating ligand and kinase domain binding modulatory phosphatidylinositol phosphates.

Structured digital abstract

- A list of the large number of protein-protein interactions described in this article is available via the MINT article ID MINT-8145255

Abbreviations

DAG, diacylglycerol; DOG, dioctanoylglycerol; eEF1A1, eukaryotic elongation factor 1A1; GST, glutathione *S*-transferase; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin; p70S6K, p70 S6 kinase; PA, phosphatidic acid; PAS, Per-Arnt-Sim; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PK, protein kinase; PL, phospholipase; PS, phosphatidylserine; PSK, protein Ser/Thr kinase; PtdIns, phosphatidylinositol; S6K, S6 kinase; TOP, terminal oligopyrimidine.

Introduction

In lower organisms, the Per-Arnt-Sim (PAS) domain is found often in environmental protein sensors involved in the perception of light intensity, oxygen partial pressure, redox potentials, voltage and certain ligands [1]. In mammals, the PAS domain is mainly found as a heterodimerization interface of transcription factors involved in dioxin signalling, the circadian clock and oxygen sensing [2–4]. We and others previously identified a novel mammalian PAS protein, alternatively called PASKIN [5] or PAS kinase [6]. PASKIN contains two PAS domains (PAS A and PAS B) and a serine/threonine kinase domain that might be regulated in *cis* by binding of so far unknown ligands to the PAS domain [7]. PASKIN shows a striking structural similarity to the bacterial oxygen sensor FixL, which contains an oxygen-binding heme group within its PAS domain [5]. Subsequent to de-repression by ligand binding, autophosphorylation in *trans* results in the 'switch-on' of the kinase domain of FixL. A similar mode of activation has been suggested also for PASKIN [6].

Protein Ser/Thr kinase (PSK)1 and PSK2, the budding yeast homologues of PASKIN, phosphorylate three translation factors and two enzymes involved in the regulation of glycogen and trehalose synthesis, thereby coordinately controlling translation and sugar flux [8]. Further experiments revealed that, under stress conditions, yeast PSK regulates translocation of UDP-glucose pyrophosphorylase 1 to the plasma membrane, where it increases cell wall glucan synthesis at the expense of glycogen storage. In the absence of PSKs, glycogen rather than glucan is produced, affecting the strength of the cell wall [9]. Two independent cell stressors have been identified to activate PSKs in yeast. Cell integrity stress (e.g. heat shock or SDS treatment) required the Wsc1 membrane stress sensor, and growth in nonglucose carbon sources (e.g. raffinose) required the AMP-dependent kinase homologue, sucrose nonfermenting 1. Although PSK2 was predominantly activated by Wsc1, PSK1 was indispensable for functioning of sucrose nonfermenting 1 [10].

In mammals, PASKIN-dependent phosphorylation inhibits the activity of glycogen synthase [11]. PASKIN has also been suggested to be required for glucose-dependent transcriptional induction of preproinsulin gene expression, which might be related to PASKIN-dependent regulation of the nuclear import of pancreatic duodenal homeobox-1 transcription factor [12,13]. However, by generating PASKIN-deficient knockout mice, we could not demonstrate any PASKIN-dependent difference in insulin gene expression or glucose tolerance [14,15]. Moreover, conflicting data were also reported on the resistance of these *Paskin* knockout mice towards high fat diet-induced metabolic syndrome [16,17].

We previously found that the eukaryotic elongation factor 1A1 (eEF1A1) is phosphorylated by PASKIN at T432 [18]. However, the role of this modification in translational control awaits further investigation. In the present study, by screening for new PASKIN kinase targets, we demonstrate that another crucial translation factor, ribosomal protein S6, can be phosphorylated by PASKIN, suggesting that PASKIN regulates protein translation not only in yeast, but also in mammals. Moreover, we identified phospholipid ligands binding to PASKIN and studied their effects on PASKIN activity.

Results

Identification of novel PASKIN kinase targets

Two approaches were applied to search for novel mammalian PASKIN targets: yeast two-hybrid and phosphorylation of peptide arrays. By yeast two-hybrid screening of a HeLa cell-derived library, we previously identified eEF1A1 as a PASKIN target [18]. In addition to novel proteins interacting with PASKIN, we also screened for novel proteins that can be phosphorylated by PASKIN. Therefore, a peptide microarray containing 1176 potential phosphoacceptor peptides was incubated with recombinant PASKIN and radioactively labelled ATP. As shown in Fig. 1A, distinct peptides were strongly phosphorylated by PASKIN (for a list of the 75 most strongly phosphorylated peptides, see Table S1). The consensus phosphoacceptor site of the 30 most strongly phosphorylated peptides was found to be similar to protein kinase (PK)A and C motifs (Fig. 1B). These data are supported by recent findings based on a combinatorial peptide library, which demonstrated a strong preference for arginine at position –3 [19]. Accordingly, from the 75 strongest hits in our screening, 70 hits indeed contain arginine three amino acids before the serine or threonine phosphoacceptor site (Table S1). Several proteins were identified more than once, either because more than one phosphoacceptor site within the same protein could be phosphorylated or because overlapping peptides containing the same phosphoacceptor site were present, or because the peptide was derived from the same site but from distinct species. Seventeen different pyruvate kinase-derived peptides, for example, were identified in this way. One of the proteins

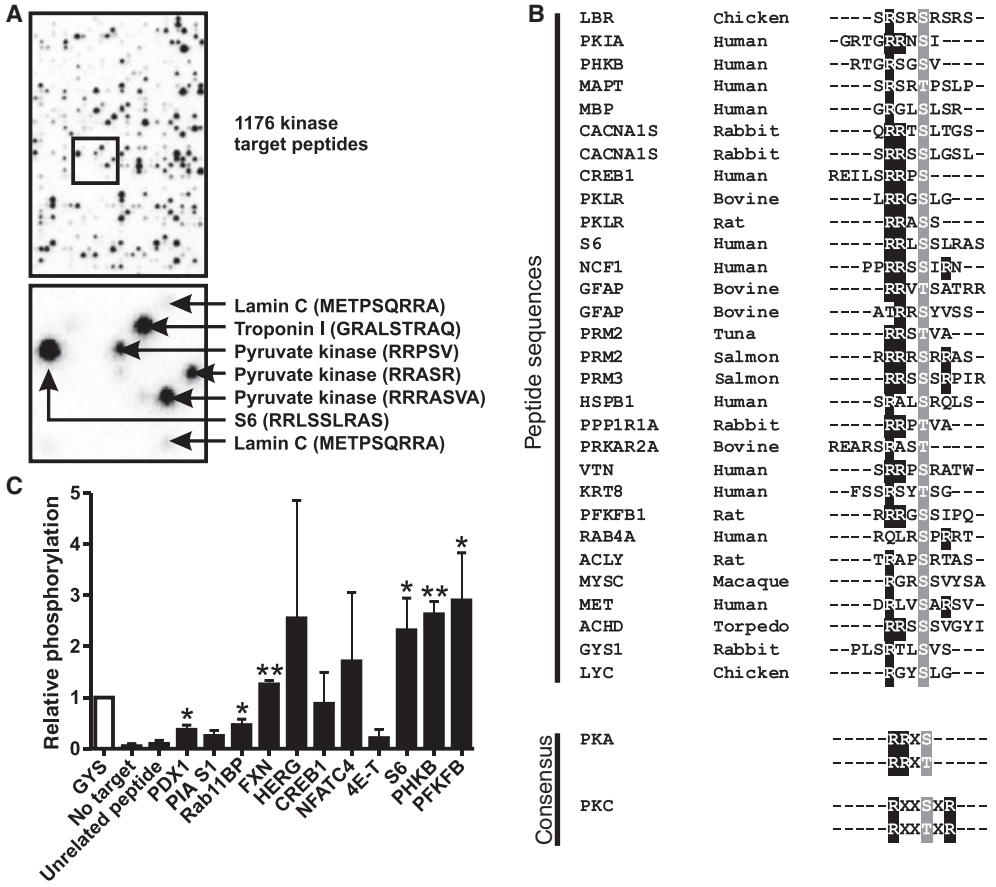

Fig. 1. Identification of novel PASKIN kinase targets. (A) Recombinant His_6-PASKIN purified from SF9 insect cells was used for *in vitro* phosphorylation of a microarray of 1176 peptides in the presence of [γ-^{33}P]ATP. The magnified inset shows an example of the results obtained after detection by phosphorimaging. (B) Peptide sequences of the 30 most phosphorylated targets and their similarities to PKA and PKC consensus motifs. (C) Target validation. Biotinylated peptides of 20 amino acids in length were incubated together with recombinant PASKIN in the presence of [γ-^{33}P]ATP, captured with streptavidin sepharose beads and quantified by liquid scintillation counting. The sequences were normalized to a glycogen synthase-derived peptide, a known target for PASKIN. A PDX-1-derived peptide, another known PASKIN target, served as second positive control. Mean ± SD values of three independent experiments are shown. Asterisks indicate statistically significant differences compared to the unrelated negative control peptide derived from activating transcription factor ATF-4 (*$P < 0.05$; **$P < 0.01$; paired *t*-test). Peptides were named: GYS, glycogen synthase; PDX1, pancreatic and duodenal homeobox 1; PIAS1, protein inhibitor of activated STAT 1; RAB11BP, RAB11-binding protein; FXN, frataxin; HERG, human ether-a-go-go related gene; CREB1, cAMP response element-binding protein; NFATC4; nuclear factor of activated T-cells c4; 4E-T, eIF4E-transporter; S6, 40S ribosomal protein S6; PHKB, phosphorylase kinase β; PFKFB, 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase. Note that some of the PASKIN target sequences, as shown in (B), can be found in several distinct proteins, leading to the partially altered designations in (C), as outlined in Table S2.

listed in Fig. 1B is glycogen synthase, which has previously been identified as a PASKIN kinase target [11]. Thus, glycogen synthase identification confirmed the feasibility of our approach and was used as a reference target protein for subsequent experiments.

To corroborate PASKIN-dependent phosphorylation of these rather short arrayed peptides, 11 of the most strongly phosphorylated candidate PASKIN kinase targets were synthesized as 20-mer peptides and used for *in vitro* phosphorylation by recombinant PASKIN (Table S2). As shown in Fig. 1C, six peptides were significantly better phosphorylated by PASKIN than the unrelated control peptide, and three of them showed an even stronger phosphorylation than the known PASKIN targets glycogen synthase and pancreatic duodenal homeobox-1 (i.e. 40S ribosomal protein S6, phosphorylase kinase β and 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase).

Ribosomal protein S6 is phosphorylated by PASKIN

Because a role for PASKIN in protein translation has been reported previously [8,18], the finding that a

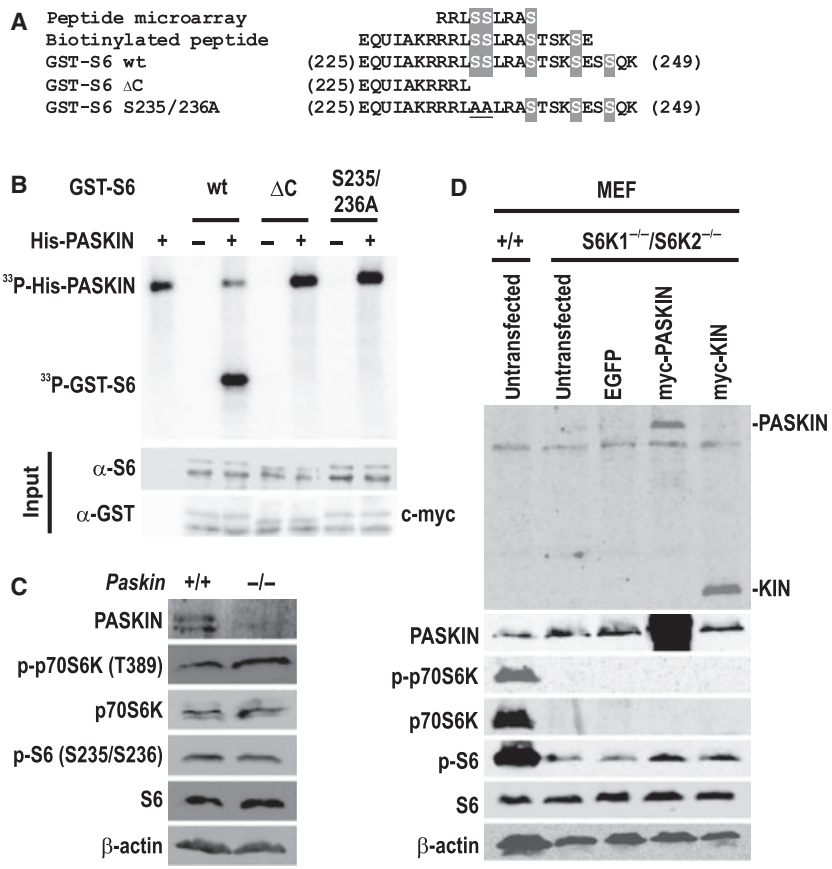

Fig. 2. Ribosomal protein S6 is phosphorylated by PASKIN. (A) Sequence comparison of the S6 peptides used in the microarray, 20-mer peptide used for the *in vitro* reactions, and recombinant GST fusion proteins purified from *E. coli*. (B) Phosphorylation reactions *in vitro* using purified His_6-PASKIN and recombinant S6 in the presence of [γ-^{33}P]ATP. Subsequent to SDS/PAGE, the phosphorylated proteins were visualized by phosphorimaging. Equal input was controlled by immunoblotting against S6 and the GST-tag. (C) Immunoblot analysis of the phosphorylation status of p70S6K and S6 in *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ MEFs. (D) Immunoblot analysis of the phosphorylation status of p70S6K and S6 in S6K1$^{-/-}$/S6K2$^{-/-}$ double-knockout MEFs after overexpression of a negative control (enhanced green fluorescent protein, EGFP), myc-PASKIN or myc-KIN. Monoclonal antibodies against myc and PASKIN were used to confirm PASKIN overexpression.

S6-derived peptide was strongly phosphorylated by PASKIN was further investigated. S6 is a target of the mammalian target of rapamycin (mTOR) signalling pathway that regulates nutrient-dependent protein translation by p70 S6 kinase (p70S6K)-mediated phosphorylation of S6 at Ser235/236 [20]. Therefore, recombinant S6 was expressed and purified either as wild-type, C-terminally truncated or Ser235/236Ala double-mutant glutathione *S*-transferase (GST) fusion protein (Fig. 2A). As shown in Fig. 2B, PASKIN phosphorylated wild-type but not truncated or serine double-mutant S6 *in vitro*, suggesting that PASKIN also targets S6 at Ser235/236.

To analyze PASKIN-dependent phosphorylation of endogenous S6 *in vivo*, we used mouse embryonic fibroblasts (MEFs) derived from either *Paskin*$^{+/+}$ wild-type or *Paskin*$^{-/-}$ knockout mice [14]. However, as shown in Fig. 2C, no difference in constitutive p70S6K or S6 phosphorylation could be detected in these cells. Because basal S6 phosphorylation by p70S6K might overcome subtle changes caused by PASKIN, we next used MEFs deficient for both genes encoding mouse p70S6K (S6K1$^{-/-}$/S6K2$^{-/-}$) [21], and transiently overexpressed full-length PASKIN or an N-terminally truncated version preserving the kinase domain in these cells. Whereas S6 total protein levels remained unchanged, phosphorylated S6 was strongly reduced in S6K1$^{-/-}$/S6K2$^{-/-}$ double-knockout MEFs (Fig. 2D). Interestingly, overexpression of myc-tagged PASKIN, or its kinase domain alone, led to increased phosphorylation of S6 at Ser235/236 (Fig. 2D). In summary, S6 is not only a new *in vitro* target, but PASKIN can also phosphorylate S6 *in vivo* and might even partially contribute to the residual S6 phosphorylation observed in p70S6K-deficient cells [22]. However, a more prominent S6 kinase function *in vivo* probably awaits the identification of the endogenous stimulus of PASKIN catalytic activity.

Autophosphorylation of recombinant PASKIN is activated by phospholipids

A possible mechanism of PASKIN activation *in vivo* might be the binding of a so far unknown ligand, as suggested previously [7]. However, no endogenous PASKIN ligand is known so far. By comparing the activity of PASKIN with PKCδ, we have obtained first indication of a potential endogenous ligand. We previously reported that both PASKIN and PKCδ phosphorylate eEF1A1 [18], and both kinases are

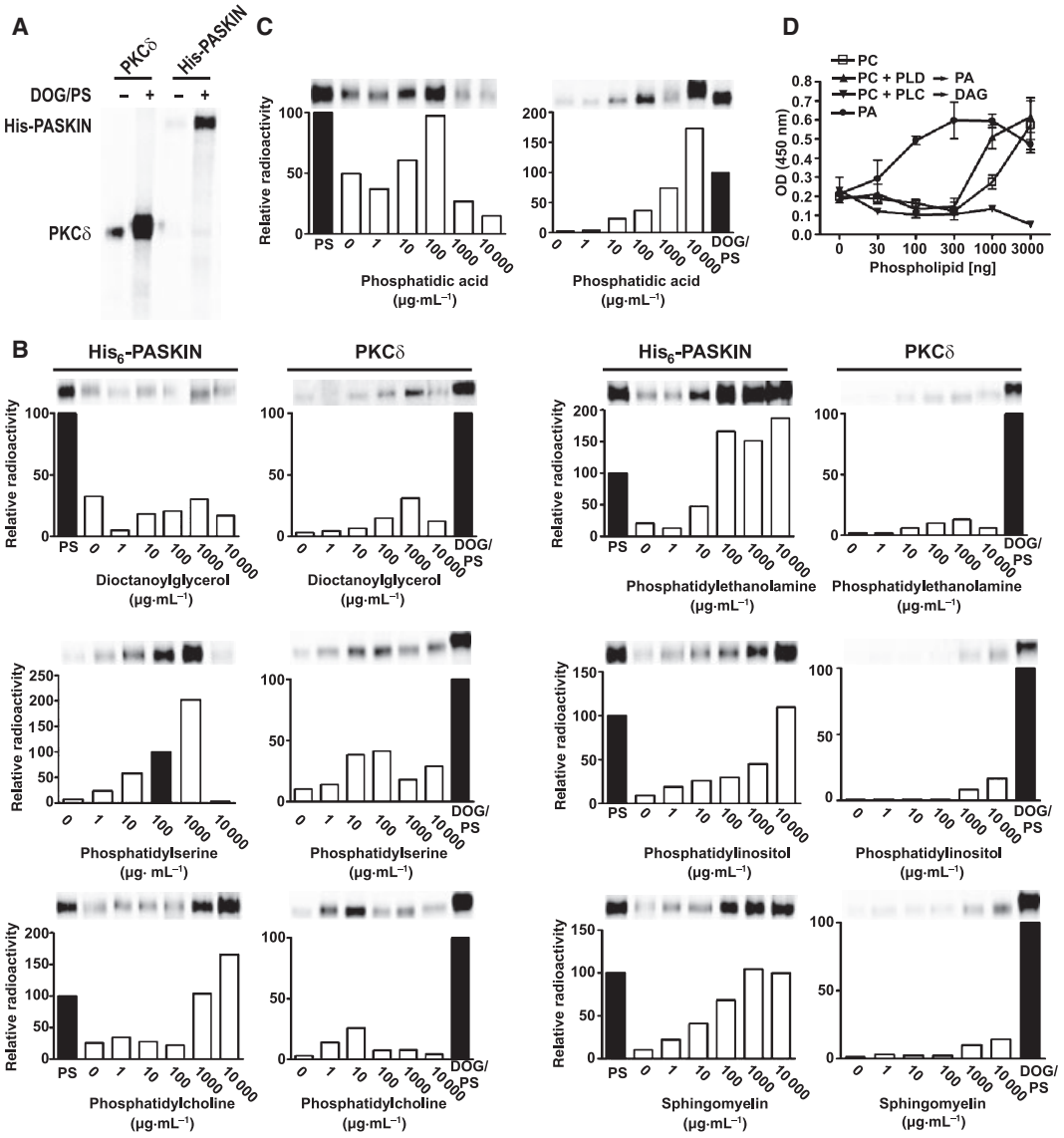

Fig. 3. Phospholipid stimulation of PASKIN autophosphorylation. (A) Lipid stimulation of PKCδ and PASKIN autophosporylation as assessed by incubating the purified recombinant proteins with DOG/PS mixtures and [γ-^{33}P]ATP. Subsequent to SDS/PAGE, the phosphorylated proteins were visualized by phosphorimaging. (B, C) Stimulation of PASKIN and PKCδ autophosphorylation by increasing amounts of the indicated phospholipids. Subsequent to SDS/PAGE, the phosphorylated proteins were visualized (upper panels) and quantified (lower panels) by phosphorimaging. The values were normalized to 100 μg·mL^{-1} PS and 10 μg·mL^{-1} DOG/100 μg·mL^{-1} PS mixtures for PASKIN and PKCδ, respectively (filled columns). (D) PLD but not PLC converts PC from a low affinity to a high affinity PASKIN ligand. Ninety-six-well plates were coated with increasing amounts of PC, followed by treatment with PLD or PLC, as indicated. Binding of 100 ng of PASKIN added to each well was detected by ELISA. Mean ± SD values of a representative experiment performed in triplicate are shown.

known to autophosphorylate themselves [6,23]. Because PKCδ kinase activity is known to be stimulated by diacylglycerol (DAG) and phosphatidylserine (PS) [24], we were interested in whether other similarities exist between PASKIN and PKCδ. Notably, a mixture of PS and DAG (used in the form of dioctanoylglycerol, DOG) not only enhanced PKCδ, but also PASKIN autophosphorylation (Fig. 3A).

To systematically analyze the lipid activation of PASKIN, all major phospholipids were compared for their effects on PASKIN and PKCδ autophosphorylation. As shown in Fig. 3B, all tested phospholipids, but not DOG alone, increased PASKIN autophosphorylation. By contrast, PKCδ autophosphorylation was induced by DOG alone, and to some extent also by PS or phosphatidylcholine (PC), although all other

phospholipids had only marginal effects on PKCδ. As shown previously [24], a mixture between DOG and PS was required to maximally induce PKCδ activity. However, combining DOG with phospholipids did not further induce PASKIN (data not shown).

The rather unselective stimulation of PASKIN activity by all tested phospholipids suggested that the core phospholipid moiety might confer PASKIN binding. Indeed, as shown in Fig. 3C, phosphatidic acid (PA) alone was sufficient to stimulate PASKIN autophosphorylation. The finding that PA but not DOG strongly bound PASKIN suggested that phospholipase (PL)D might target PASKIN by converting phospholipids into PA. To directly demonstrate this assumption, 96-well plates were coated with constant amounts (1 μg) of PC. After treatment with PLD or PLC, increasing amounts of PASKIN were added and detected by ELISA. As shown in Fig. 3D, PASKIN bound with clearly higher affinity to PA than to PC. However, lipid binding was restored when PC was treated with PLD (generating PA) but not with PLC (generating DAG).

Inositol phosphorylation determines the affinity of phosphatidylinositol (PtdIns) interaction with PASKIN

The experiments described above suggested that an isolated phosphate group such as in PA is necessary for maximal PASKIN–lipid interaction. Because PtdIns with varying numbers of phosphate groups belong to the most important cellular lipid signalling molecules, we next investigated whether the number and location of the phosphate groups on the inositol ring affect their interaction with PASKIN. Therefore, dot blots containing mono-, di- and tri-phosphorylated PtdIns were incubated with recombinant PASKIN and immunodetected using a monoclonal antibody derived against PASKIN. Unexpectedly, although unphosphorylated PI showed only relatively low PASKIN binding, this interaction was strongly increased by the presence of a single phosphate group in PtdIns(4)P, and reduced again when two or three phosphate groups were present in PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, respectively (Fig. 4A). This finding was corroborated by using dot blots with increasing amounts of all possible PtdIns-phosphates: PASKIN dose-dependently bound PtdIns-monophosphates better than PtdIns-diphosphates, and nonphosphorylated or tri-phosphorylated PtdIns bound PASKIN only weakly (Fig. 4B, left). Similar results were obtained with autophosphorylated PASKIN (Fig. 4B, right), suggesting that PASKIN phosphorylation status does not interfere with selective PtdIns-monophosphate binding.

To localize the region responsible for PtdIns-monophosphate binding, four different fragments of PASKIN (Fig. 4C, left) were expressed and purified as His_6-tagged fusion proteins. However, only the kinase domain of PASKIN bound PtdIns-monophosphates (Fig. 4C, right), rather than the previously suggested ligand-binding PAS domain (data not shown). We next aimed to determine the effects of differently phosphorylated PtdIns on PASKIN autophosphorylation. As shown in Fig. 4D, autophosphorylation was dose-dependently enhanced by all three PtdIns-monophosphates, whereas especially high concentrations of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 even inhibited autophosphorylation, establishing a structure–function relationship between kinase domain–lipid interaction and kinase activity.

PtdIns-monophosphate-dependent regulation of PASKIN target phosphorylation

Because PtdIns-monophosphates stimulated PASKIN autophosphorylation, we were interested in whether they could also stimulate phosphorylation of the PASKIN targets S6 and eEF1A1. Therefore, wild-type and phosphoacceptor site mutant recombinant S6 and eEF1A1 were used for PASKIN *in vitro* phosphorylation reactions in the presence of differently phosphorylated PtdIns-phosphates. As shown in Fig. 5, PASKIN autophosphorylation was again stimulated by all three PtdIns-monophosphates but inhibited by PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. Although the phosphoacceptor site mutant S6 and eEF1A1 GST fusion proteins remained unphosphorylated, their wild-type counterparts were phosphorylated by PASKIN. Unexpectedly, both S6 and eEF1A1 target phosphorylation was inhibited by PtdIns-phosphates. The more phosphate groups the inositol ring carries, the stronger the PASKIN target protein phosphorylation was inhibited. However, nonphosphorylated PtdIns did not significantly change the target phosphorylation efficiency.

Discussion

In the present study, we identified various novel potential PASKIN substrates by peptide microarray phosphorylation, including glycogen synthase that was known before to be phosphorylated by PASKIN [11]. Thus, the repetitive identification of this PASKIN target confirms, at least partially, the validity of the peptide array approach. Other peptides derived from proteins involved in glycogen metabolism included

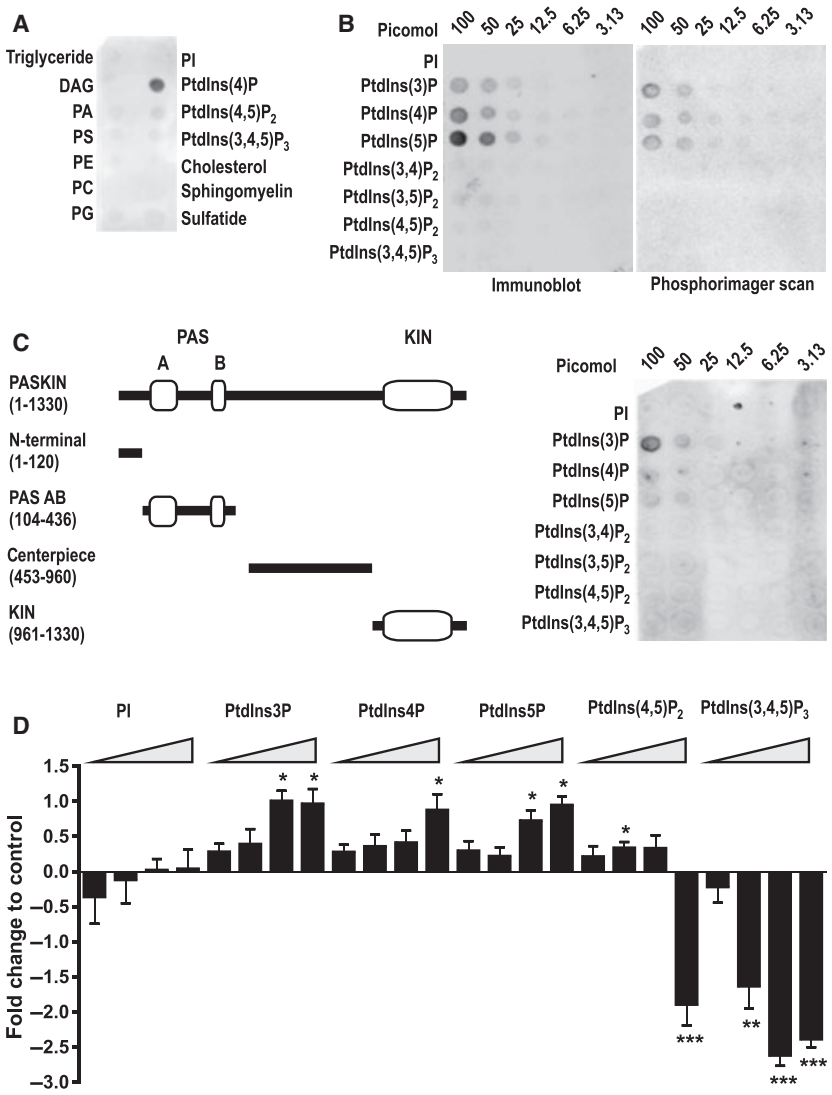

Fig. 4. Preferential PASKIN binding to (and activation by) PtdIns-monophosphates. (A) Recombinant His_6-PASKIN protein was allowed to bind to the indicated lipids immobilized on a membrane, and subsequently detected using PASKIN antibodies. (B) PASKIN dose-dependently bound preferably PtdIns-monophosphates. PASKIN was either detected by immunoblotting (left panel) or by phosphorimaging after autophosphorylation in the presence of [γ-^{33}P]ATP (right panel). (C) Fragments of PASKIN were expressed in *E. coli* and purified as His_6-tagged fusion proteins (left panel). Subsequent to binding to the lipid dot blots and detection using a His-tag antibody, only the kinase (KIN) domain of PASKIN was found to interact with PtdIns-monophosphates (right panel). (D) His_6-PASKIN autophosphorylation was mainly stimulated by the presence of the PtdIns-monophosphates. *In vitro* phosphorylation reactions in the presence of [γ-^{33}P]ATP and the indicated synthetic diC8 PtdIns (3.16 μM, 10 μM, 31.6 μM and 100 μM) were separated by SDS/PAGE and quantified by phosphorimaging. Values were expressed relative to lipid-free control reactions and are represented as the mean ± SD of four independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *t*-test).

phosphorylase kinase, inhibitor of protein phosphatase 1 and yeast glycogen phosphorylase (Table S1). The involvement of PASKIN in the regulation of glycogen synthesis was demonstrated previously by showing that both mammalian and yeast glycogen synthases, as well as yeast UDP-glucose pyrophosphorylase, are known phosphorylation targets of mammlian PASKIN and yeast PSK1 and PSK2, respectively [8,11]. However, although Ser640 was the main PASKIN kinase target residue of mammalian glycogen synthase [11], the peptides phosphorylated by PASKIN on the microarray contained Ser3 and Ser7 but not Ser640. Of note, a Ser640Ala mutation did not completely prevent phosphorylation [11]. Therefore, our data suggest that PASKIN might phosphorylate Ser3 and/or Ser7 of glycogen synthase in addition to Ser640.

Two peptides phosphorylated by PASKIN were derived from enzymes involved in glycolysis: pyruvate kinase and 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase 1 (Table S1). Obviously, the coordination of glycolysis, gluconeogenesis and glycogen synthesis appears to be physiologically meaningful, and hence it is tempting to speculate that PASKIN is involved in the regulation of all of these metabolic pathways. However, pyruvate kinase could not be confirmed as a PASKIN target using purified full-length pyruvate kinase GST fusion proteins in *in vitro* assays (data not shown).

Interestingly, S6 was among the peptides phosphorylated by PASKIN and this phosphorylation could be confirmed on the full-length protein level. Together with the previously reported eEF1A1 phosphorylation [18], this finding provides additional evidence that PASKIN is involved in mammalian protein translation. The most important and best characterized S6 kinases are the mTOR-dependent p70 S6-kinases that sequentially phosphorylate all five phosphorylatable

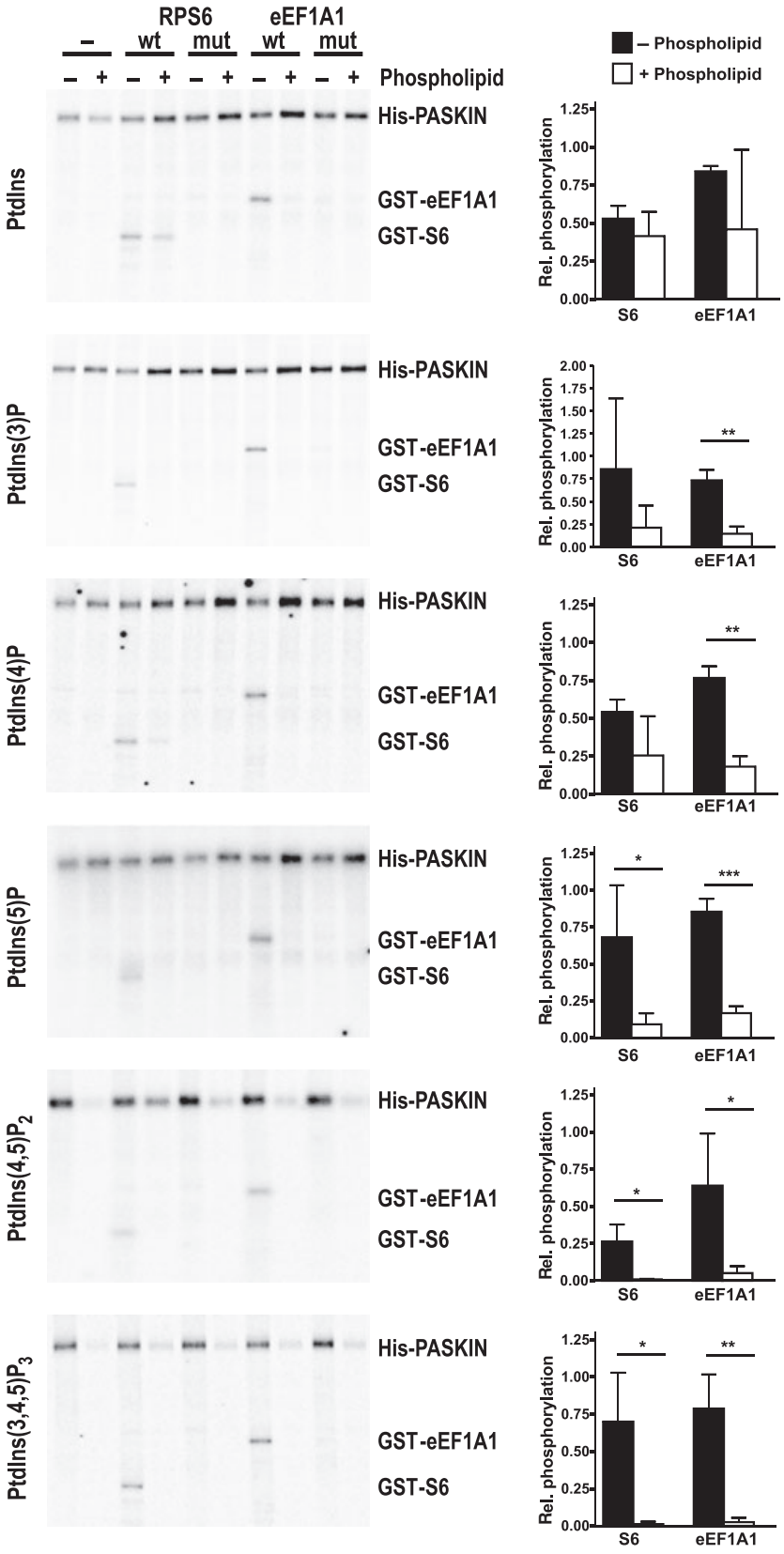

Fig. 5. *In vitro* target phosphorylation of His_6-PASKIN is reduced in presence of PtdIns. Recombinant His_6-PASKIN purified from Sf9 insect cells was used to *in vitro* phosphorylate recombinant GST fusion proteins with wild-type S6, with the nonphosphorylatable double-mutant S235/236A, with eEF1A1, or with its nonphosphorylatable T432A mutant, in the presence of [γ-^{33}P]ATP and PtdIns phosphates (100 μM) as indicated. Subsequent to separation by SDS/PAGE, protein phosphorylation was viusalized (left panel, representative images) and quantified (right panel) by phosphorimaging. His_6-PASKIN autophosphorylation without lipid and target (first lane from the left) was used for intra-assay normalization of the values. Columns represent the mean ± SD values of three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *t*-test).

serines of S6, starting with S236 and S235 (i.e. the same sites as shown in the present study for PASKIN) followed by S240, S244 and S247 [25]. A second family of S6 kinases are p90 ribosomal S6 kinases that phosphorylate S6 upon mitogenic stimulation at the same sites as PASKIN [22]. Phosphorylation of S6 by p70S6K has long been considered to increase protein translation by selectively enhancing the translation of 5′-terminal oligopyrimidine (TOP) mRNAs, a subset of mRNAs containing an oligopyrimidine tract in their 5′-UTRs. Of note, the 5′-TOP mRNAs code for ribosomal proteins and translation factors, including PASKIN targets S6 and eEF1A1 [26]. However, S6 phosphorylation and increased 5′-TOP mRNA translation might be coincidental rather than causally related [27] and, according to a newer hypothesis, might even negatively influence translation if the phosphorylation of S6 is considered as an inhibitory feedback signal [28]. However, no significant difference in global [^{35}S]-Met incorporation could be observed in *Paskin*$^{-/-}$ MEFs (data not shown).

On the basis of the known functions of the PASKIN-related FixL oxygen sensor in bacteria and the PASKIN orthologues in yeast, and considering the lack of any obvious phenotype in *Paskin* knockout mice kept under normal housing conditions, it is tempting to assume that PASKIN has a ligand-mediated sensor function that becomes apparent under currently ill-defined stress situations [17]. However, only artificial but no endogenous PASKIN ligands have been reported to bind the PAS domain and lead to the derepression of the kinase domain-dependent autophosphorylation [7]. In the present study, we identified phospholipids as the first biologically relevant PASKIN ligands. Apparently, the presence of a charged phosphate moiety is required for stimulation of PASKIN kinase activity, and PLD (but not PLC) can convert phospholipids from low into high-affinity PASKIN ligands. However, we currently do not know whether PASKIN is a target of intracellular PLD cell signalling.

Unexpectedly, PtdIns-monophosphates were found to be the best ligands of PASKIN, with clearly higher affinities than PtdIns-diphosphates or PtdIns-triphosphate. PtdIns-binding domains have been reported to display either well-defined 3D folds [29], or rather unstructured regions with basic (for binding of the phosphate groups) and hydrophobic residues, such as in the noncanonical pleckstrin homology domain of Tiam1 [30]. We identified a lysine rich region, spanning from Lys1019 to Lys1034 of PASKIN, which shares characteristic features with noncanonical pleckstrin homology domains, including a double-lysine motif (Lys1031/1032). However, mutation and deletion analyses of this putative binding region did not affect lipid binding by PASKIN (data not shown). Thus, it is difficult to predict the PtdIns-monophosphate binding site within the PASKIN kinase domain and further work will be necessary to identify the specific residues involved in lipid binding.

Although PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are involved in signalling processes at the plasma membrane, PtdIns-monophosphates are more abundant in intracellular membrane structures such as the Golgi apparatus and endosomes [31]. Within these structures, PtdIns-monophosphates are involved in sorting and signalling because the concentrations and localization of differentially phosphorylated PtdIns can change rapidly [29]. Therefore, it might be possible that PtdIns-monophosphates not only regulate PASKIN activity, but also its subcellular localization. This hypothesis needs further investigation but is dependent on the prior identification of the specific environmental conditions that regulate PASKIN function.

As might be expected, we found a direct correlation between ligand affinity and PASKIN autophosphorylation efficiency. However, the kinase domain rather than the PAS domain was found to bind the PtdIns-phosphates. This finding might explain why the activation of PASKIN-dependent S6 and eEF1A1 target phosphorylation failed to comply with our initial expectations: PASKIN autophosphorylation was not directly related to target phosphorylation. However, the results obtained in the present study are consistent with a recent study reporting that PASKIN kinase activity is independent of activation loop phosphorylation [19]. Thus, the original model of autophosphorylation-dependent kinase activity needs to be revised, and the functional meaning of PASKIN autophosphorylation remains to be elucidated.

In conclusion, the *in vitro* data obtained in the present study suggest the existence of downstream effector functions of mammalian PASKIN similar to those known from yeast: the coordination between energy flux and translation. With the identification of endogenous small molecule activators of PASKIN, we have obtained the first indication of the upstream regulators of PASKIN activity. It will be interesting to examine how these regulators affect the downstream processes mediated by PASKIN.

Experimental procedures

Plasmids

All cloning work was carried out using Gateway technology (Invitrogen, Carlsbad, CA, USA). The human PASKIN

cDNA containing plasmids pENTR4-hPASK and pENTR4-hKIN, as well as plasmids for recombinant expression of full length His_6-PASKIN, PASKIN truncations and eukaryotic elongation factor 1A1, have been reported previously [18]. pENTR4-hPASK and pENTR4-hKIN were recombined into pcDNA3.1/c-myc-DEST [32] using LR recombinase (Invitrogen) to generate pcDNA3.1/c-myc-hPASK and pcDNA3.1/c-myc-hKIN for c-myc-tagged expression of PASKIN or its kinase domain, respectively, in mammalian cells. Human ribosomal protein S6 (IRAUp969B0849D6; Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany) was cloned into pENTR4 using primers 5′-TTATGTCGACATGAAGCTGAACAT-3′ (forward) and 5′-TACGTGCGGCCGCTTATTTCTGACTGGATTCAGA CTTAG-3′ (reverse), respectively, or 5′-TACGTGGCGGC CGCTTAAAGTCTGCGTCTCTTCGC-3′ to introduce a stop codon after residue L234. The PCR products were ligated into the *Sal*I and *Not*I restriction sites. The S6 S235/236A double mutant was produced with primers 5′-GCGAAGAGACGCAGGCTAGCCGCTCTGCGAGC TTCTAC-3′ and 5′-GTAGAAGCTCGCAGAGCGGCT AGCCTGCGTCTCTTCGC-3′ by Pfu polymerase-based site-directed mutagenesis (Stratagene, La Jolla, CA, USA). For expression as GST-tagged fusion proteins, pENTR4 based plasmids with the different S6 constructs were recombined into pDEST15 using LR recombinase.

Purification of recombinant proteins

Recombinant proteins were purified as described previously [18]. Briefly, full-length PASKIN was purified from Sf9 cells using the Bac-to-Bac Baculovirus expression system (Invitrogen). GST-tagged fusion constructs and His_6-tagged PASKIN fragments were expressed in arabinose inducible BL21 *Escherichia coli*. Recombinant proteins were purified by FPLC (BioLogic DuoFlow; Bio-Rad, Hercules, CA, USA) using HiTrap Chelating HP and GSTrap FF columns (GE Healthcare, Milwaukee, WI, USA), respectively. The kinase activity of purified recombinant PASKIN was verified by autophosphorylation assays.

Kinase assays

His_6-PASKIN or PKCδ (Invitrogen) were incubated with or without 2 μg of recombinant target proteins in kinase buffer (25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol) for 20 min in the presence of 3 μCi [γ-^{33}P]ATP (Hartmann Analytic, Brunswick, Germany). Proteins were separated by SDS/PAGE and analyzed by phosphorimaging of the dried gels (Molecular Imager FX; Bio-Rad) using QUANTITY ONE software (Bio-Rad). Lipids (Sigma, St Louis, MO, USA or Fluka, Buchs, Switzerland) were dissolved in $CHCl_3$, aliquotted in test tubes and the $CHCl_3$ evaporated under a stream of nitrogen. Lipids were then resuspended in kinase assay master mixes by thorough vortexing. PtdIns present in the phosphorylation reactions were obtained from Echelon Biosciences (Salt Lake City, UT, USA) as synthetic diC8-lipids and added to the reactions from 1 mM aequous stock solutions to the final concentrations indicated.

Peptide microarrays

Peptide microarrays were phosphorylated with recombinant PASKIN in accordance with the manufacturer's instructions (Pepscan, Lelystad, The Netherlands). In brief, 50 μL of a solution containing 500 ng recombinant PASKIN, 50 mM Hepes (pH 7.4), 20 mM $MgCl_2$, 10% glycerol, 300 μCi·mL^{-1} [γ-^{33}P]ATP, 0.01% (v/v) Brij-35 and 0.01 mg·mL^{-1} BSA was added to the glass slide, covered with a glass coverslip and incubated at 30 °C for 2 h in a humidified incubator. After incubation, the coverslip was removed with 1% Triton X-100 in NaCl/P_i and the glass slide was washed twice with 1% Triton X-100 in 2 M NaCl and twice with water by over-head shaking, air-dried and analyzed by phosphorimaging (Bio-Rad).

Phosphorylation of biotinylated peptides

PASKIN phosphorylation reactions were performed as described above in the presence of N-terminally biotinylated 20-mer target peptides (JPT Peptide Technologies, Berlin, Germany) at a final concentration of 200 μM. The reactions were stopped by adding SDS to 0.5% final concentration and heating at 95 °C for 5 min. Streptavidin sepharose beads (25 μL; GE Healthcare) and 500 μL of 100 mM Tris–HCl (pH 8.0) were added and incubated for 30 min at 4 °C. The beads were washed three times with 500 μL of a buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 400 mM NaCl, 0.1% Nonidet P-40 and once with 500 μL of 100 mM Tris (pH 8.0). Phosphorylation of the beads was quantified by liquid scintillation counting (Packard Tri-Carb 2900TR; Perkin Elmer, Boston, MA, USA).

Cell culture, transfections and immunoblotting

MEF cells were generated from *Paskin*$^{+/+}$ and *Paskin*$^{-/-}$ mice [14] at embryonic day 14. *S6K1*$^{-/-}$/*S6K2*$^{-/-}$ double-knockout MEFs were kindly provided by G. Thomas and S. C. Kozma (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). MEF cells were cultivated in DMEM (Sigma) supplemented with 10% fetal bovine serum (Invitrogen) up to passage 12, suggesting that they immortalized spontaneously. MEFs were transiently transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Thirty-six hours post-transfection, cells were harvested and whole cell lysates were generated by heating the cells in 1% SDS for 5 min at 95 °C. After SDS/PAGE and

immunoblotting, the primary antibodies used were: human PASKIN (Pierce, Rockford, IL, USA); mouse PASKIN, phospho-S6 (S235/236), S6 kinase and phospho-S6 kinase (T389) (Cell Signaling Technology, Beverly, MA, USA); S6 (Bethyl Laboratories, Montgomery, TX, USA); β-actin and GST-tag (Sigma); and His-tag (Novagen, Madison, WI, USA).

Lipid binding assays

Interactions between PASKIN and lipids were measured by an ELISA-based assay, as described previously [33]. Briefly, 96-well plates (Sarstedt, Nümbrecht, Germany) were coated overnight with phospholipids dissolved in methanol, followed by blocking with 3% BSA in NaCl/P_i for 1 h. Purified His_6-PASKIN (100 ng) was diluted in kinase buffer and allowed to bind for 1 h at 30 °C. After three washing steps (0.3% Tween-20 in NaCl/P_i), bound PASKIN was detected by anti-PASKIN mAb6, followed by secondary goat anti-(mouse IgG) horseradish peroxidase-conjugated sera (Pierce) using the 3,3′,5,5′-tetramethylbenzidine substrate kit (Pierce). The peroxidase reaction was stopped by adding H_2SO_4 (final concentration of 1 M) and A_{450} was determined using a microplate reader (Digiscan; Asys Hitech, Eugendorf, Austria). For PL experiments, phospholipid-coated 96-well plates were treated with 0.2 units of PLC or PLD (Sigma), diluted in reaction buffer (120 mM $CaCl_2$, 300 mM sodium acetate, pH 5.6) for 1 h at room temperature.

Lipid binding arrays

Membranes spotted with phospholipids were obtained from Echelon Biosciences (P-6002, P-6100) and used in accordance with the manufacturer's instructions. Generally, 1 μg of protein diluted in 1% skimmed dry milk in NaCl/Tris was allowed to bind to spotted phospholipids for 16–20 h at 4 °C. Binding was detected using primary antibodies as indicated and horseradish peroxidase-coupled secondary antibodies for enhanced chemiluminescence detection (Pierce).

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Supporting information

The following supplementary material is available:
Table S1. Rank order of the 75 most strongly phosphorylated PASKIN kinase targets on the peptide microarray.
Table S2. Sequences of the eleven biotinylated peptides tested for phosphorylation by recombinant PASKIN.

This supplementary material can be found in the online version of this article.

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