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p38γ regulates the localisation of SAP97 in the cytoskeleton by modulating its interaction with GKAP

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Activation of the p38 MAP kinase pathways is crucial for the adaptation of mammalian cells to changes in the osmolarity of the environment. Here we identify SAP97/hDlg, the mammalian homologue of the *Drosophila* tumour suppressor Dlg, as a physiological substrate for the p38γ MAP kinase (SAPK3/p38γ) isoform. SAP97/hDlg is a scaffold protein that forms multiprotein complexes with a variety of proteins and is targeted to the cytoskeleton by its association with the protein guanylate kinase-associated protein (GKAP). The SAPK3/p38γ-catalysed phosphorylation of SAP97/hDlg triggers its dissociation from GKAP and therefore releases it from the cytoskeleton. This is likely to regulate the integrity of intercellular–junctional complexes, and cell shape and volume in response to osmotic stress.

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Introduction

Cells respond to changes in the physical and chemical properties of the environment by altering many cellular functions. These environmental changes include alterations in the concentrations of nutrients, growth factors, cytokines and cell-damaging agents, but also physical stimulation mediated by changes in the osmolarity in the medium. When exposed to hyperosmotic stress, eukaryotic cells shrink due to the efflux of water from the cell (Morris *et al*, 2003). For cellular integrity and homeostasis to be maintained under these conditions, cells employ adaptive responses to restore cell volume (O'Neill, 1999) and reinforce the cytoskeletal architecture (Di Ciano *et al*, 2002). Mammalian cells respond to changes in the osmolarity by activating signalling pathways that involve the activation of p38 mitogen-activated protein kinases (MAPKs) (Kyriakis and Avruch, 2001) which are critical for long-term cellular adaptation to prolonged hyperosmotic exposure. The changes triggered by this pathway include alterations in gene transcription (Sheikh-Hamad *et al*, 1998; Garmyn *et al*, 2001) and post-translational modification of cytoskeletal remodelling proteins (Landry and Huot, 1999; Bustamante *et al*, 2003). Although all four p38 MAPKs (p38α, p38β, SAPK3/p38γ and SAPK4/p38δ) are activated in mammalian cells in response to hyperosmotic stress, the activation of SAPK3/p38γ is particularly rapid and strong compared to other p38s (Goedert *et al*, 1997a; Sabio *et al*, 2004). Here we identify a novel physiological substrate for SAPK3/p38γ, which is likely to play an important role in the adaptive response to osmotic stress.

To identify new substrates of SAPK3/p38γ, we have exploited a characteristic of this kinase that is unique among all MAPK family members, namely the presence of a carboxyl-terminal sequence which can dock with the PDZ domains of different proteins. Moreover, the phosphorylation of PDZ-domain-containing proteins by SAPK3/p38γ is dependent on this interaction (Hasegawa *et al*, 1999; Sabio *et al*, 2004), suggesting that such proteins are likely to be its physiological substrates. One of these proteins is the synapse-associated protein 97 (SAP97/hDlg), the mammalian homologue of the *Drosophila* tumour suppressor gene *dlg*, which is a member of a family of membrane-associated guanylate kinase (GK) homologues (MAGUKs) (Garner *et al*, 2000). SAP97/hDlg is found at the pre- and post-synaptic density in neuronal cells and at the region of cell–cell contacts along the epithelial lateral membrane. SAP97/hDlg has also been localised at the neuromuscular junction in muscle and at the lymphocyte immune synapse (Muller *et al*, 1995; Reuver and Garner, 1998; Leonoudakis *et al*, 2001). Structurally, SAP97/hDlg is composed of three N-terminal PDZ domains, followed by an Src homology 3 (SH3) domain and a GK-like region (Garner *et al*, 2000). Functionally, SAP97/hDlg is a scaffolding protein that assembles multicomponent protein complexes; thereby facilitating signal transduction, and it has also been implicated in maintaining cell adhesion and cell polarity (Caruana, 2002; Humbert *et al*, 2003).

An important unresolved issue, with regard to the ability of SAP97/hDlg to build multi-component protein complexes, is which kinases and phosphatases act directly on it to regulate its association with different binding partners. In this study, we have found that SAPK3/p38γ binds directly to two PDZ domains of SAP97/hDlg and phosphorylates it *in vivo* in response to cellular stress. Moreover, we show that the

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phosphorylation of SAP97/hDlg by SAPK3/p38γ regulates its association to the GK-associated protein (GKAP) and its localisation in the cytoskeleton.

Results

Phosphorylation of SAP97/hDlg by SAPK3/p38γ depends on their interaction

Immunolocalisation studies showed that in HeLa, PC12 and SH5-SY5Y cells SAPK3/p38γ and SAP97/hDlg both had the same diffuse cytoplasmic and nuclear localisation (Figure 1A), and in PC12 along neuritic processes (Figure 1A VII–IX), indicating a possible interaction between them in these cellular compartments. This was confirmed by the finding that SAPK3/p38γ co-immunoprecipitated with SAP97/hDlg (Figure 1B). Since SAP97/hDlg contains three PDZ domains, we first assessed the importance of the C-terminus of SAPK3/p38γ on the binding to SAP97/hDlg. We therefore examined whether these proteins co-immunoprecipitated from extracts of human embryonic kidney (HEK)293 cells transfected with either SAPK3(FL) (full length) or SAPK3(ΔC) (lacking the last four amino acids). As predicted, endogenous SAP97/hDlg only co-immunoprecipitated with FL SAPK3/p38γ (Figure 1B). These results indicate that the last four amino acids of SAPK3/p38γ are essential for its association with the PDZ domain of SAP97/hDlg. Secondly, to determine which SAP97-PDZ domain was responsible for the interaction, cells were transiently transfected with DNA constructs encoding the different SAP97-PDZ domains together with SAPK3/p38γ. We found that SAPK3/p38γ interacted with PDZ domains 1 and 3 of SAP97/hDlg in pulldown and co-immunoprecipitation experiments (Figure 1C).

Moreover, the phosphorylation of SAP97/hDlg by SAPK3/p38γ *in vitro* was dependent on the extreme C-terminus of SAPK3/p38γ. SAPK3(ΔC) phosphorylated SAP97/hDlg very

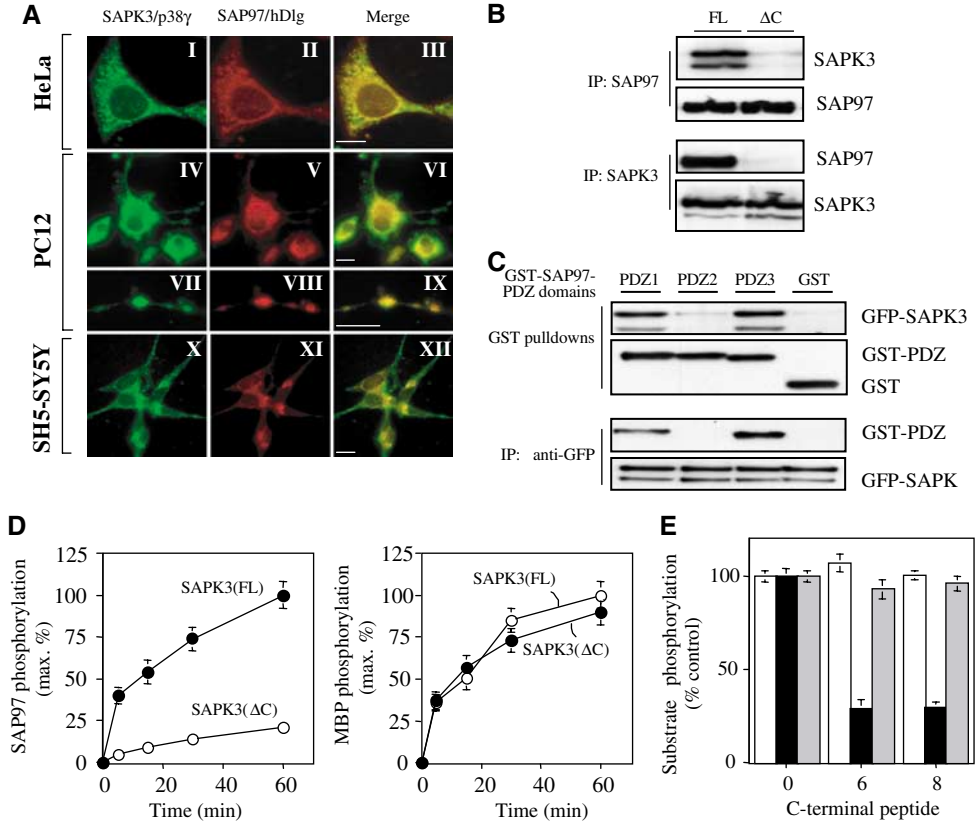


Figure 1 Phosphorylation of SAP97/hDlg by SAPK3/p38γ depends on the interaction of these two proteins. (**A**) HeLa cells (I, II, III), differentiated PC12 (IV–IX) and differentiated SH5-SY5Y neuroblastoma (X, XI, XII) were stained with anti-SAPK3 or anti-SAP97 antibody, and subjected to fluorescence microscopy. SAPK3/p38γ and SAP97/hDlg are shown in green and red, respectively. In merged images, the co-localised signal is shown in yellow. Scale bar, 10 μM. (**B**) Co-immunoprecipitation of SAPK3/p38γ with SAP97. HEK293 cells were transfected with GFP-SAPK3(FL) or GFP-SAPK3 (lacking the last four amino acids (ΔC)). Endogenous SAP97 in the lysates was immunoprecipitated, and immunoblotted with anti-SAPK3 or anti-SAP97 antibodies. GFP-SAPK3 was immunoprecipitated using an anti-GFP antibody, and immunoprecipitates immunoblotted with anti-SAP97 or anti-SAPK3 antibodies. (**C**) Interaction of SAPK3/p38γ with SAP97PDZ domains. HEK293 cells were transfected with GFP-SAPK3(FL) and either GST, GST-SAP97(PDZ1), GST-SAP97(PDZ2) or GST-SAP97(PDZ3). After transfection the cells were lysed and GST-fusion proteins purified by affinity chromatography on GSH-Sepharose beads. The GFP-SAPK3 was immunoprecipitated using an anti-GFP antibody. The proteins were immunoblotted using an anti-SAPK3/p38γ antibody to detect GFP-SAPK3/p38γ, or anti-GST antibody to detect expression of GST-SAP97 fusion proteins. (**D**) Phosphorylation of SAP97 by SAPK3/p38γ is dependent on the carboxy-terminal four amino acids of the kinase. GST-SAP97 or MBP, both at 1 μM, were phosphorylated for the times indicated with 2.0 U/ml of either GST-SAPK3(FL) or GST-SAPK3(ΔC). The results are shown as the mean ± s.e.m. of four experiments. (**E**) GST-SAP97 (filled bars) or MBP (open bars), each at 1 μM, were incubated for 30 min at room temperature with synthetic peptides (300 μM) corresponding to the C-terminal six (PKETAL) or eight (RVPKETAL) amino acids of rat SAPK3. GST-SAPK3/p38γ (black bars) or GST-SAPK4/p38δ (grey bars) were added to 0.2 U/ml and the reactions initiated with Mg[γ-^{32}P]ATP. Substrate phosphorylation is plotted as a percentage of that measured in the absence of each peptide. Results in (**E**) are shown as the mean ± s.e.m. for triplicate determinations from a single experiment.

poorly, though it phosphorylated myelin basic protein (MBP; a protein devoid of PDZ domains) as well as full-length SAPK3(FL) (Figure 1D). Also, pre-incubation of SAP97/hDlg with synthetic peptides corresponding to the C-terminal six or eight amino acids of SAPK3/p38γ prevented phosphorylation of SAP97/hDlg by SAPK3/p38γ, but not by SAPK4/p38δ (Figure 1E).

Phosphorylation of SAP97/hDlg after cellular stresses

We investigated whether other members of the MAPK family could phosphorylate SAP97/hDlg and SAP102, another PDZ-domain-containing member of the MAGUK family. We compared initial rates of phosphorylation by different MAPK family members *in vitro* and showed that SAP97/hDlg was phosphorylated efficiently by SAPK3/p38γ or SAPK4/p38δ, whereas p38α, p38β, c-jun N-terminal kinase (JNK)1-3 or extracellular signal regulated kinase-2 (ERK2) only phosphorylated SAP97/hDlg much more slowly. In contrast, SAP102 was a very poor substrate for all the MAPKs tested (Table I).

SAP97/hDlg was phosphorylated *in vitro* by SAPK3/p38γ and SAPK4/p38δ at six residues. Three of these (Ser^{431}, Ser^{442} and Ser^{447}) are located between PDZ domains 2 and 3, whilst residues Ser^{122}, Ser^{158} and Thr^{209} are located N-terminal to PDZ domain 1 (Figure 2A). To examine whether endogenous SAP97/hDlg became phosphorylated when cells were exposed to hyperosmotic stress that triggers the activation of p38s, we generated five different phospho-specific antibodies that recognise the major sites phosphorylated by SAPK3/p38γ or SAPK4/p38δ in SAP97/hDlg (Supplementary Figure 1). Endogenous SAP97/hDlg from HEK293 cells became phosphorylated at Ser^{158}, Thr^{209}, Ser^{431} and Ser^{442} after osmotic shock, whereas UV-C radiation only induced phosphorylation at Ser^{158} and Ser^{442} (Figure 2B). Ser^{122} was phosphorylated in unstimulated cells and phosphorylation did not increase in response to osmotic stress. The antibody did not recognised SAP97/hDlg in which Ser^{122} was mutated to Ala (Figure 2C) confirming that this residue is constitutively phosphorylated in cells.

In order to obtain information about the kinase(s) responsible for SAP97/hDlg phosphorylation, we incubated cells with SB203580 and/or PD184352, prior to exposure to UV-C or osmotic shock. SB203580 is a relatively specific inhibitor of p38α and p38β activity, whilst PD184352 is a potent inhibitor of the classical ERK1/2 pathway and the ERK5 pathway (Davies *et al*, 2000; Mody *et al*, 2001). Neither compound had a significant effect on the phosphorylation of any of the phosphorylation sites of endogenous SAP97/hDlg by these cellular stresses (Figure 2B). In contrast, SB203580 prevented the phosphorylation of Hsp27 a well known *in vivo* substrate of MAPKAP-K2 which is activated downstream of p38α (Supplementary Figure 2). These results were consistent with phosphorylation of SAP97/hDlg being mediated by SAPK3/p38γ, since UV-C and sorbitol treatment did not activate SAPK4/p38δ in these cells (Supplementary Figure 2), the other MAPK family member that phosphorylates SAP97/hDlg efficiently *in vitro* and is not inhibited by SB203580. We also incubated cells with the cell permeant peptide TatSAPK3C which contains the last nine residues of SAPK3/p38γ fused to the cell-membrane transduction domain of the human immunodeficiency virus-type 1 (HIV-1) Tat protein. This peptide specifically blocks the phosphorylation of PDZ domain-containing proteins by SAPK3/p38γ in intact cells by preventing the association of the kinase with the PDZ domain of the substrate (Sabio *et al*, 2004). We treated the cells with wild-type TatSAPK3C(WT) peptide or with the non-interacting mutant TatSAPK3C(AA) peptide, in which the last four residues corresponding to the C-terminal of SAPK3/p38γ are mutated to Ala before exposure to osmotic shock. Phosphorylation of endogenous SAP97/hDlg at all residues induced by exposure to sorbitol was completely suppressed by Tat-SAPK3C(WT), but not by TatSAPK3C(AA) (Figure 2D). The Tat-SAPK3C peptide did not prevent the activation of SAPK3/p38γ or p38α, and did not affect the phosphorylation of MAPKAP-K2 (Supplementary Figure 2). These results indicate that the Tat-SAPK3C peptide prevents SAP97/hDlg phosphorylation by blocking its association with SAPK3/p38γ and again suggest that this kinase is responsible for the phosphorylation of SAP97/hDlg under these conditions.

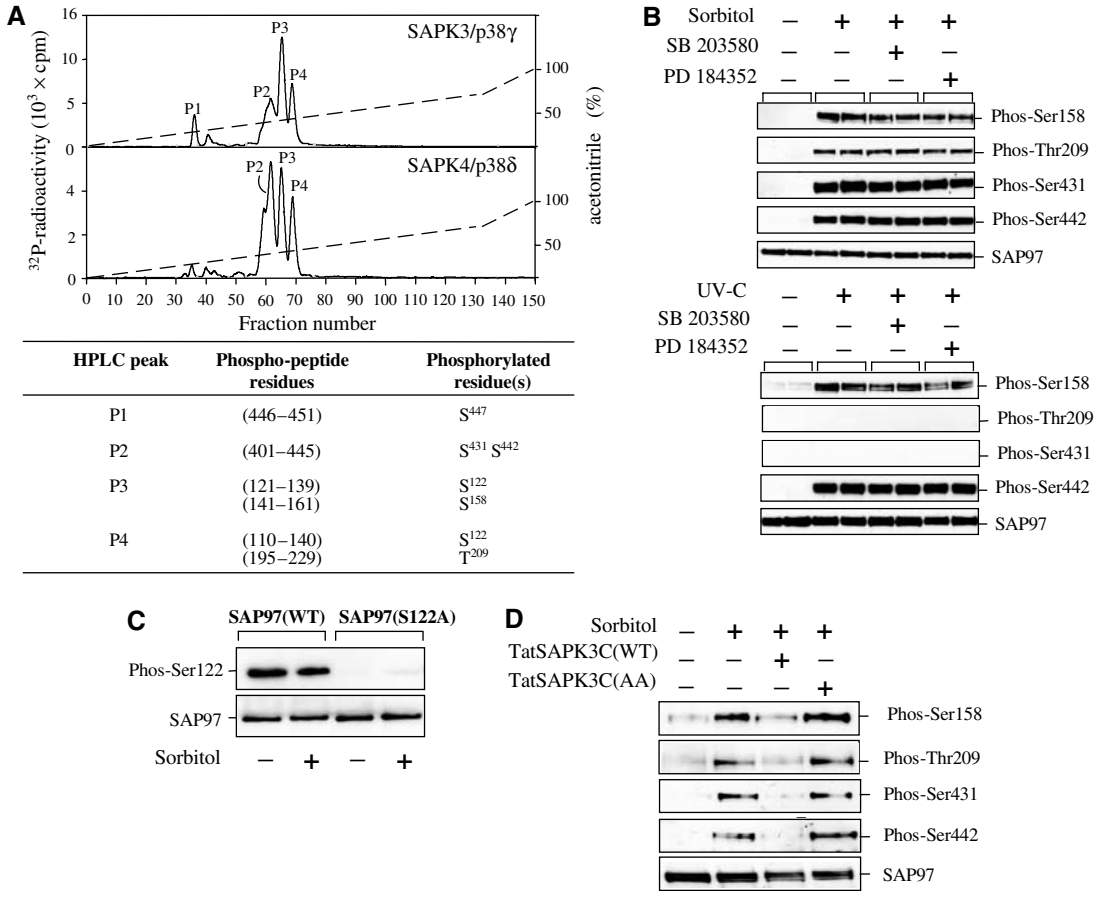
Generation of SAPK3/p38γ and SAPK4/p38δ knockout mice

To further investigate whether SAPK3/p38γ mediates SAP97/hDlg phosphorylation in cells we generated mice deficient in

Table I Comparison of substrate specificities of different MAP kinase family members

Kinase (0.5 U/ml)	Rates of phosphorylation relative to myelin basic protein (MBP)			
	SAP97	SAP102	SAP90	MBP
SAPK3/p38γ	100±3	3.1±0.1	100±4	100
SAPK4/p38δ	42±2	0.1	74±5	100
p38α	8.5±0.9	0.1	2.8±0.2	100
p38β	6.3±0.7	0.1	3.1±0.2	100
MAPK2/ERK2	1.7±0.2	0.1	20±5	100
Kinase (0.1 U/ml)	Rates of phosphorylation relative to the activating transcription factor-2 (ATF2)			
	SAP97	SAP102	SAP90	ATF2
SAPK3/p38γ	100±2.8	3.1±0.1	100±5	100
JNK2α	1.0±0.2	0.2	6.2±1	100
JNK3	0.5±0.02	1.0±0.1	4.0±0.5	100
JNK1α	0.23±0.1	0.1	0.4±0.1	100

Each enzyme was assayed under initial rate conditions as described previously (Cuenda *et al*, 1997). The final concentration of the different substrates was 1 μM.



HPLC peak	Phospho-peptide residues	Phosphorylated residue(s)
P1	(446–451)	S^{447}
P2	(401–445)	S^{431} S^{442}
P3	(121–139) (141–161)	S^{122} S^{158}
P4	(110–140) (195–229)	S^{122} T^{209}

Figure 2 Hyperosmotic stress induces phosphorylation of endogenous SAP97/hDlg. (**A**) Identification of the sites on SAP97/hDlg phosphorylated *in vitro* by SAPK3/p38γ or SAPK4/p38δ. Rat GST-SAP97 was incubated for 1 h at 30°C with Mg[γ-^{32}P]ATP in the presence of 0.5 U/ml of SAPK3/p38γ or SAPK4/p38δ, and subjected to SDS–PAGE. The phosphorylated SAP97 was excised from the gel, digested with trypsin and the peptides separated by chromatography. The column was developed with an acetonitrile gradient (broken line) and ^{32}P-radioactivity is shown in full line. The phosphopeptides P1–P4 are indicated. To identify the residue phosphorylated in P2, it was subdigested with the protease Asp-N to give a smaller phospho-peptide (residues 427–445). P3 and P4 are a mixture of two peptides, each phosphorylated at a single residue. All residues were identified by a combination of techniques MALDI-TOF, Q-TOF, MS/MS, solid phase sequencing and phospho-amino-acid analysis. (**B**) Phosphorylation of SAP97/hDlg in HEK293 cells after cellular stress. Cells were incubated for 1 h with or without 10 μM SB203580 and/or 5 μM PD184352, then exposed for 15 min to 0.5 M sorbitol or to UV-C radiation (200 J/m^2), followed by a 30 min incubation. Endogenous SAP97/hDlg was immunoprecipitated from 1–5 mg of cell lysate, the pellets immunoblotted using an antibody that recognises SAP97 phosphorylated at S^{158} (Phos-Ser158), T^{209} (Phos-Thr209), S^{431} (Phos-Ser431), S^{442} (Phos-Ser442) and an antibody that recognises unphosphorylated and phosphorylated SAP97 equally well. The lanes in this panel are duplicates. (**C**) HEK293 were transfected with SAP97 WT or GST-SAP97 mutant in which S^{122} has been mutated to Ala. GST-SAP97 was immunoprecipitated from 50 μg of lysate, and the pellets were immunoblotted using Phos-Ser122 antibody. (**D**) HEK293 cells were incubated for 1 h with or without 400 μM TatSAPK3C(WT) or TatSAPK3C(AA) peptide, and then exposed for 15 min to 0.5 M sorbitol. The immunoprecipitated SAP97/hDlg was immunoblotted as above.

SAPK3/p38γ, SAPK4/p38δ or both kinases. To generate these knockout mice we used the targeting constructs shown in Figure 3. ES cells containing the targeted genes were identified by the appearance of 8 kb band on Southern blots when using 3′ probe, in addition to the 11 kb WT band (Figure 3A). The heterozygous SAPK4/p38δ ES cells containing the targeted genes were identified by the appearance of a 7.3 kb band on Southern blots when using 5′ probe, in addition to the 20 kb WT band (Figure 3B). Full details of how the mouse knockouts were generated are previously described (Wiggin *et al*, 2002). Both, the SAPK3/p38γ and SAPK4/p38δ, knockout mice were viable and fertile and of normal appearance and had no obvious health problems when kept under stress free and specific-pathogen-free conditions. Since SAPK3/p38γ is localised to chromosome 22q (Goedert *et al*, 1997b) and SAPK4/p38δ to chomosome 6p (Herbison *et al*, 1999), SAPK3/p38γ and SAPK4/p38δ double knockout were produced by intercrossing mice with single knockouts of each protein kinase. Genotypes of the single and double knockouts were confirmed by PCR (Figure 3A and B). Like the single knockout, the double knockout was viable and fertile and had no obvious health problems.

SAPK3/p38γ was expressed at similar levels in the WT and in the SAPK4/p38δ knockout mouse embryonic fibroblasts (MEF) but was not detectable in the SAPK3/p38γ or double knockout MEF (Figure 3C). SAPK4/p38δ was at similar levels in the WT and in the SAPK3/p38γ knockout MEF, but was not detectable in the SAPK4/p38δ or double knockout MEF. The levels of expression of p38α and p38β in either the single or the double knockout were similar to those for WT MEF (Figure 3C).

On the other hand, treatment of MEF with cellular stresses caused the activation of the p38 pathway in these cells. In

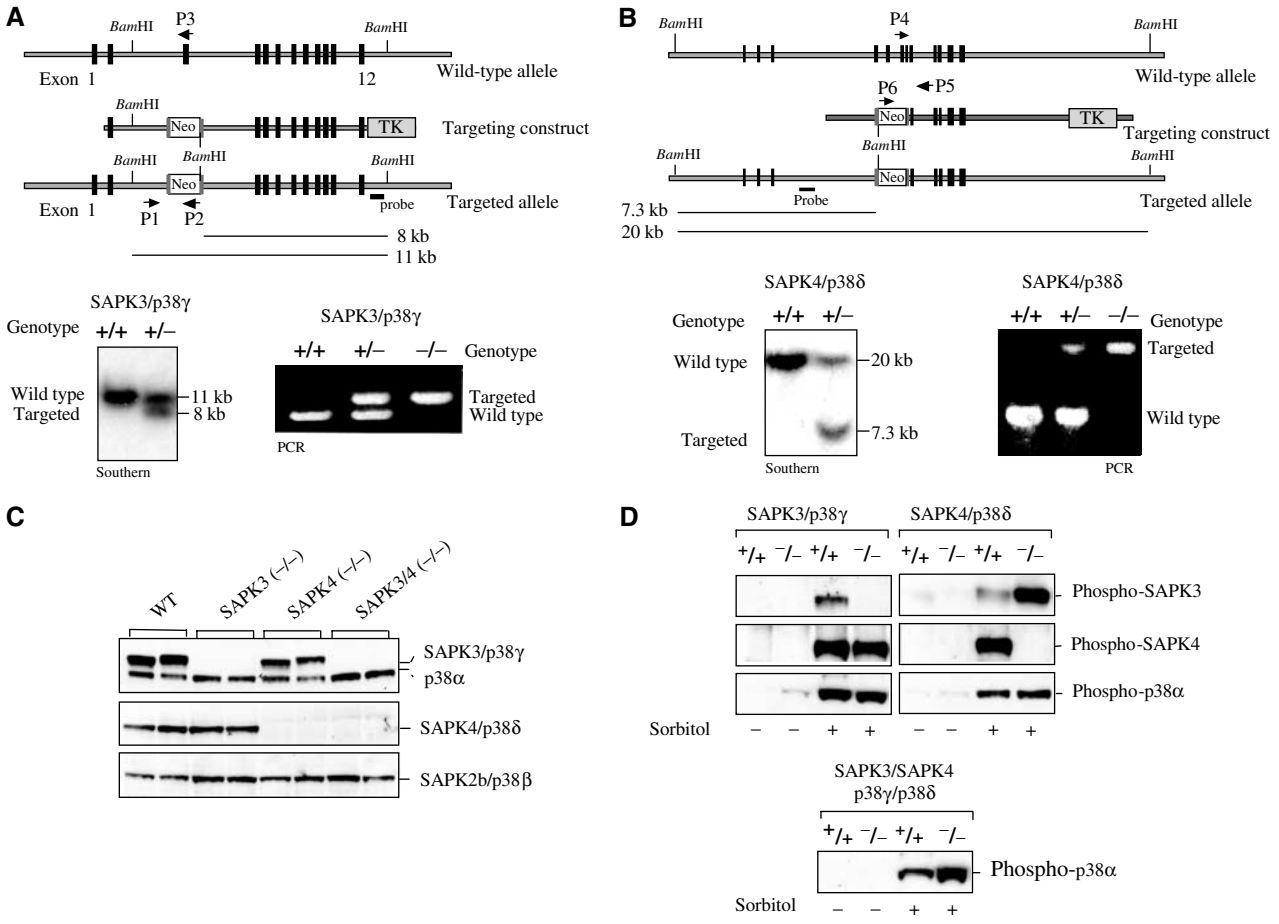


Figure 3 Generation of SAPK3/p38γ and SAPK4/p38δ knockouts. Diagram illustrating the targeting vector for the knockout of SAPK3/p38γ (**A**) and SAPK4/p38δ (**B**). The black boxes represent exons, the positions of the probes and the PCR primers used for genotyping are indicated by white boxes and black arrows, respectively. Genomic DNA purified from indicated ES cell lines were digested with *Bam*HI, electrophoresed on a 1% agarose gel, transferred to nitrocellulose for Southern blotting. Genomic DNA purified from tail biopsy sample was used as a template for PCR, electrophoresed on a 1% agarose gel and examined by ethidium bromide staining. (**C**) Lysates from MEF WT, SAPK3/p38γ, SAPK4/p38δ and SAPK3/4 double knockout (30–50 μg of protein) were immunoblotted with antibodies that recognise specifically each p38. The lanes in this panel are duplicates. (**D**) MEF SAPK3/p38γ, SAPK4/p38δ and SAPK3/4 double knockout were exposed for 15 min to 0.5 M sorbitol. To examine the activation of p38s, SAPK3/p38γ or SAPK4/p38δ were immunoprecipitated from 2 mg of cell lysates, and immunoblotted with the p38α phospho-specific antibody that also recognises phosphorylated SAPK3/p38γ and SAPK4/p38δ. Alternatively, 50 μg of cell lysates was immunoblotted with the same phospho-specific antibody to detect active p38α.

WT, SAPK3/p38γ(−/−), SAPK4/p38δ(−/−) and double knockout cells, p38α was phosphorylated after exposure to osmotic shock (sorbitol), whereas SAPK3/p38γ was activated in WT and SAPK4/p38δ knockout cells, and SAPK4/p38δ was phosphorylated in WT and SAPK3/p38γ knockout cells (Figure 3D).

Phosphorylation of SAP97/hDlg in MEF

We carried out experiments using MEF from these mice in combination with the use of different kinase inhibitors. In WT MEF, osmotic shock caused detectable phosphorylation of endogenous SAP97/hDlg at Ser^{158}, Thr^{209} and Ser^{442}, but not Ser^{431} (Figure 4). When cells were pre-incubated with SB203580 and/or PD184352, neither compound had a significant effect on the phosphorylation of any of the sites of endogenous SAP97/hDlg by sorbitol (Figure 4). Similar results were obtained in SAPK4/p38δ(−/−) fibroblasts, except that the phosphorylation of Thr^{209} was slightly enhanced compared to the WT (Figure 4B).

In SAPK3/p38γ(−/−) cells the phosphorylation of SAP97/hDlg at Ser^{158} was completely lost indicating that this kinase is solely responsible for the phosphorylation of this site (Figure 4A). However, surprisingly endogenous SAP97/hDlg was still phosphorylated at residues Thr^{209} and Ser^{442} in SAPK3/p38γ(−/−) cells, although Ser^{442} phosphorylation was slightly reduced compared to the WT. Moreover, in contrast to WT MEFs, phosphorylation at Ser^{442} was now blocked if SAPK3/p38γ(−/−) cells were pre-incubated with SB203580 (Figure 4A). However, phosphorylation at Thr^{209} was unaffected by SB203580 in SAPK3/p38γ(−/−) cells. On the other hand, in double knockout MEF cells deficient in both SAPK3/p38γ and SAPK4/p38δ, the phosphorylation of Thr^{209} was greatly reduced, and the phosphorylation at Ser^{442} partially reduced. Phosphorylation at both sites disappeared completely when these cells were pre-treated with SB203580 (Figure 4C). As expected from the results with SAPK3/p38γ(−/−) MEF, the phosphorylation of Ser^{158} did not occur in the double knockout cells, even in the absence of SB203580. The compound PD184352 did not block the phosphorylation of endogenous SAP97/hDlg by these cellular stresses in either WT, single or double knockout MEF (Figure 4).

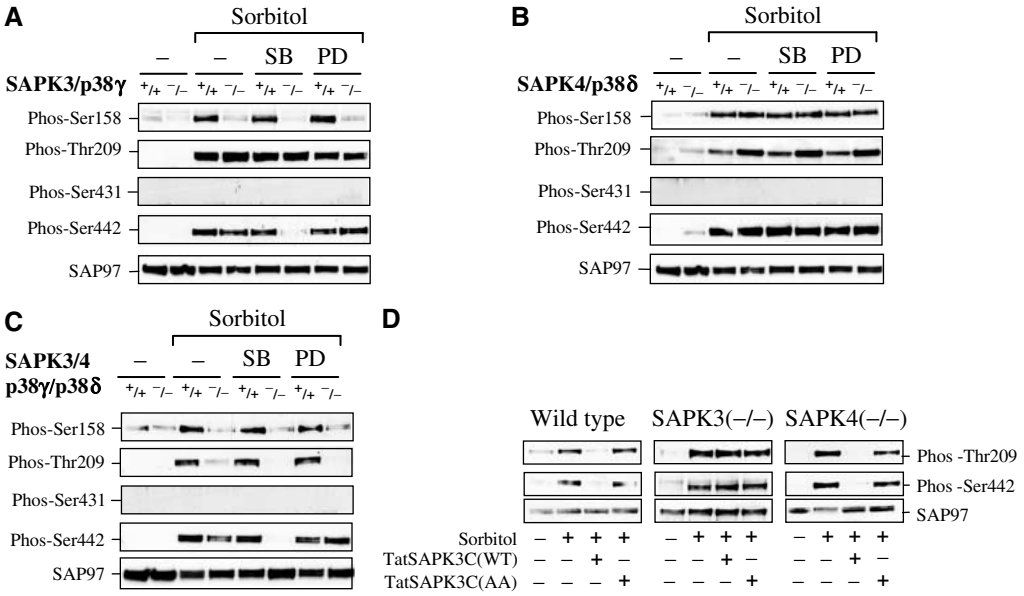


Figure 4 Phosphorylation of endogenous SAP97/hDlg in mouse embryonic fibroblasts. MEF from WT, SAPK3/p38γ(−/−) (**A**), SAPK4/p38δ(−/−) (**B**) or SAPK3/4 double knockout mice (**C**) were incubated for 1 h with or without 10 μM SB203580 or 5 μM PD184352, then exposed for 15 min to 0.5 M sorbitol. Endogenous SAP97 was immunoprecipitated from 1–5 mg of cell lysate, the pellets immunoblotted using an antibody that recognises SAP97 phosphorylated at S^{158} (Phos-Ser158), T^{209} (Phos-Thr209) or S^{442} (Phos-Ser442), or with an antibody that recognises both unphosphorylated and phosphorylated SAP97. (**D**) Mouse embryonic fibroblasts from WT, SAPK3/p38γ(−/−) or SAPK4/p38δ(−/−) were incubated for 1 h with or without 400 μM TatSAPK3C(WT) or TatSAPK3C(AA), and then exposed for 15 min to 0.5 M sorbitol. The immunoprecipitated SAP97 was immunoblotted as above.

In order to obtain more information about the protein kinases acting on Thr^{209} and Ser^{442}, we pre-incubated WT, SAPK3/p38γ(−/−) and SAPK4/p38δ(−/−) MEF with either TatSAPK3C(WT) or with the non-interacting TatSAPK3C(AA) peptide prior to stimulation with sorbitol. In WT and SAPK4/p38δ(−/−) cells, phosphorylation of endogenous SAP97/hDlg at Thr^{209} and Ser^{442}, was abolished by TatSAPK3C(WT) (Figure 4D). However, in SAPK3/p38γ(−/−) cells, phosphorylation of SAP97/hDlg at both residues, was unaffected by either TatSAPK3C(WT) or TatSAPK3C(AA) peptide (Figure 4D). Taken together our results demonstrate that in WT cells, the phosphorylation of Ser^{158}, Thr^{209} and Ser^{442} is mediated by SAPK3/p38γ. In SAPK3/p38γ(−/−) cells SAP97/hDlg is still phosphorylated at Thr^{209} by SAPK4/p38δ and at Ser^{442} mainly by p38α or p38β MAPK. Our results are a clear example of functional compensation by highly related protein kinases, when one member is not expressed.

Regulation of SAP97/hDlg-GKAP interaction by phosphorylation after hyperosmotic stress

Since SAP97/hDlg is a scaffolding protein implicated in the assembly of macromolecular protein complexes we studied whether its phosphorylation affects the binding to other proteins. For this, we first examined the association of SAP97/hDlg with different proteins, such as CASK and GKAP, and the PDZ binding kinase (PBK) and SAPK3/p38γ (Figure 5A), whose association with different domains of SAP97/hDlg is well characterised (Gaudet *et al*, 2000; Wu *et al*, 2000; Lee *et al*, 2002). In co-immunoprecipitation experiments only the interaction of SAP97/hDlg with the protein GKAP is affected by osmotic shock in HEK293 cells (Figure 5A). The anti-SAP97 antibody immunoprecipitated more than 90% of SAP97/hDlg and GKAP from PC12 cells and approx. 50% from HEK293 cells lysates (Figure 5B), suggesting that almost all endogenous GKAP is associated with SAP97/hDlg in these cells. We then checked whether the association of GKAP with SAP97/hDlg *in vitro* is dependent on the phosphorylation state of SAP97/hDlg. Recombinant SAP97/hDlg either unphosphorylated or phosphorylated by SAPK3/p38γ, was incubated with GKAP. After immunoprecipitation of GKAP the unphosphorylated SAP97/hDlg was detected in the immunocomplex whereas more than 90% of the phospho-SAP97 remained in the supernatant. Moreover, when SAP97 was immunoprecipitated, GKAP was pulled down with the unphosphorylated but not the phosphorylated form of SAP97 (Figure 5C), indicating that the association between these two proteins was regulated by the phosphorylation of SAP97/hDlg.

Endogenous GKAP and SAP97/hDlg were also associated in unstimulated SH-SY5Y neuroblastoma and in PC12 cells, but dissociated following exposure of cells to osmotic shock. However, the interaction between endogenous GKAP and SAP97/hDlg was unaffected when cells had been exposed to UV-C, suggesting that phosphorylation of SAP97/hDlg at residues Thr^{209} and/or Ser^{431}, which do not become phosphorylated after exposure to UV-C, may be important in regulating interaction with GKAP (Figure 5D). To investigate this possibility we co-transfected cells with WT GKAP and either WT GST-SAP97 or different GST-SAP97 mutants, in which each of the phosphorylation sites were mutated individually to Ala. The association of GKAP with the different SAP97/hDlg mutants was checked by co-immunoprecipitation from untreated or osmotically shocked cells (Figure 5E). As expected, WT SAP97/hDlg interacted with GKAP in untreated cells but not following osmotic shock, whereas every SAP97/hDlg phosphorylation site mutant, except for S122A which is constitutively phosphorylated in cells (Figure 2C),

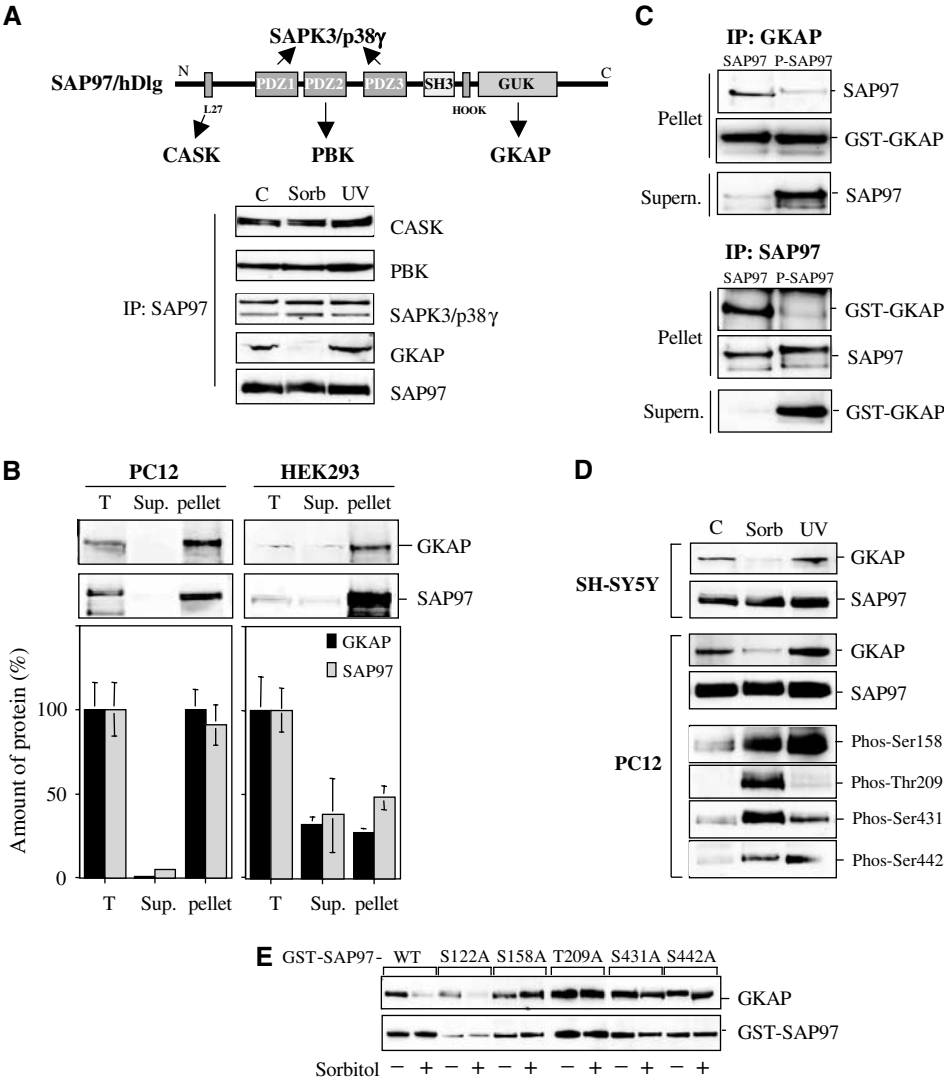


Figure 5 Association of SAP97/hDlg with the protein GKAP is regulated by phosphorylation. (**A**) Structural organisation of SAP97/hDlg, indicating that the protein CASK binds to the L27 domain, SAPK3/p38γ to the PDZ1 and PDZ3 domains, PBK to PDZ2 and GKAP to the GK domain. HEK293 cells were transfected with PBK or SAPK3/p38γ. Cells were left unstimulated or exposed for 15 min to 0.5 M sorbitol or to UV-C radiation (200 J/m^2), followed by a 30 min incubation, and endogenous SAP97/hDlg was immunoprecipitated from 0.2–15 mg of cell lysate. The pellets were immunoblotted using antibodies that recognise CASK, PBK, SAPK3/p38γ, GKAP or SAP97/hDlg. (**B**) Endogenous SAP97/hDlg was immunoprecipitated from 0.2 mg of undifferentiated PC12 or 15 mg of HEK293 cell lysate. The pellet and 100 μg of protein from both, total lysates (T) (as loading control) or the supernatants (Sup.) were immunoblotted, using antibodies that recognise GKAP or SAP97/hDlg (upper panel). Quantification of the amount of protein detected is shown in the lower panel. (**C**) Recombinant SAP97 (500 ng) unphosphorylated or phosphorylated with SAPK3/p38γ was incubated with recombinant GST-GKAP (500 ng). After 10 min at 4°C, GKAP or SAP97 were immunoprecipitated, and the washed pellets and supernatants immunoblotted with an anti-GKAP antibody or anti-SAP97 antibody. (**D**) Endogenous SAP97 was immunoprecipitated from 0.5 mg of SH-SY5Y or undifferentiated PC12 lysate treated as in (A). The pellets were immunoblotted with the antibodies indicated in the figure. (**E**) HEK293 cells were transfected with WT GKAP and either WT GST-SAP97 or different GST-SAP97 mutants, and then exposed for 15 min osmotic shock (0.5 M sorbitol). Expressed SAP97 was immunoprecipitated from 0.5 mg of protein lysates and the pellets were immunoblotted with the antibodies indicated.

bound to GKAP in either untreated or shocked cells (Figure 5E). These results indicate that phosphorylation of SAP97/hDlg at all four residues regulated by osmotic shock, is required to prevent the binding of GKAP and that lack of phosphorylation in any one site is sufficient to maintain the interaction between the two proteins.

SAP97/hDlg recruitment to the cytoskeleton though GKAP

It has been shown that GKAP associates with intermediate filaments and neurofilaments (Hirao *et al*, 2000) and can also recruit PDZ domain-containing proteins to the plasma membrane in transfected cells (Takeuchi *et al*, 1997). We therefore examined whether the association of SAP97/hDlg with GKAP alters its localisation in the cell. Both over-expressed and endogenous SAP97/hDlg were present in all cellular compartments, whereas GKAP was expressed mainly in the cytoskeletal fraction. Interestingly, the localisation at the cytoskeleton of SAP97/hDlg full length (FL) but not SAP97(ΔGK), which is a SAP97/hDlg mutant lacking the GK domain and therefore unable to bind GKAP (Figure 6 and Supplementary Figure 4), is increased significantly in cells transfected with GKAP, suggesting that this protein recruits SAP97/hDlg to the cytoskeleton (Figure 6A).

Moreover we have found that the amount of SAP97/hDlg, but not GKAP, in the cytoskeletal fraction of HEK293 and PC12 cells decreased greatly after exposure to hyperosmotic shock (Figure 6B). In addition, the amount of cytokeratin co-immunoprecipitated with either endogenous or transfected SAP97/hDlg also decreased upon osmotic stress, but not the cytokeratin that immunoprecipitated with GKAP (Figure 6C). On the other hand, cytokeratin did not co-immunoprecipitate with SAP97(ΔGK) (Figure 6C). These results suggest that the decrease in the amount of SAP97/hDlg bound to the cytoskeletal fraction upon hyperosmotic stress is due to dissociation from GKAP caused by the SAPK3/p38γ-catalysed phosphorylation of SAP97/hDlg. If this hypothesis is correct, then phosphorylation site mutants of SAP97/hDlg that do not dissociate from GKAP upon osmotic stress should remain in the cytoskeletal fraction after osmotic stress. To verify this, cells were transfected with WT SAP97/hDlg or different SAP97/hDlg phosphorylation site mutants.

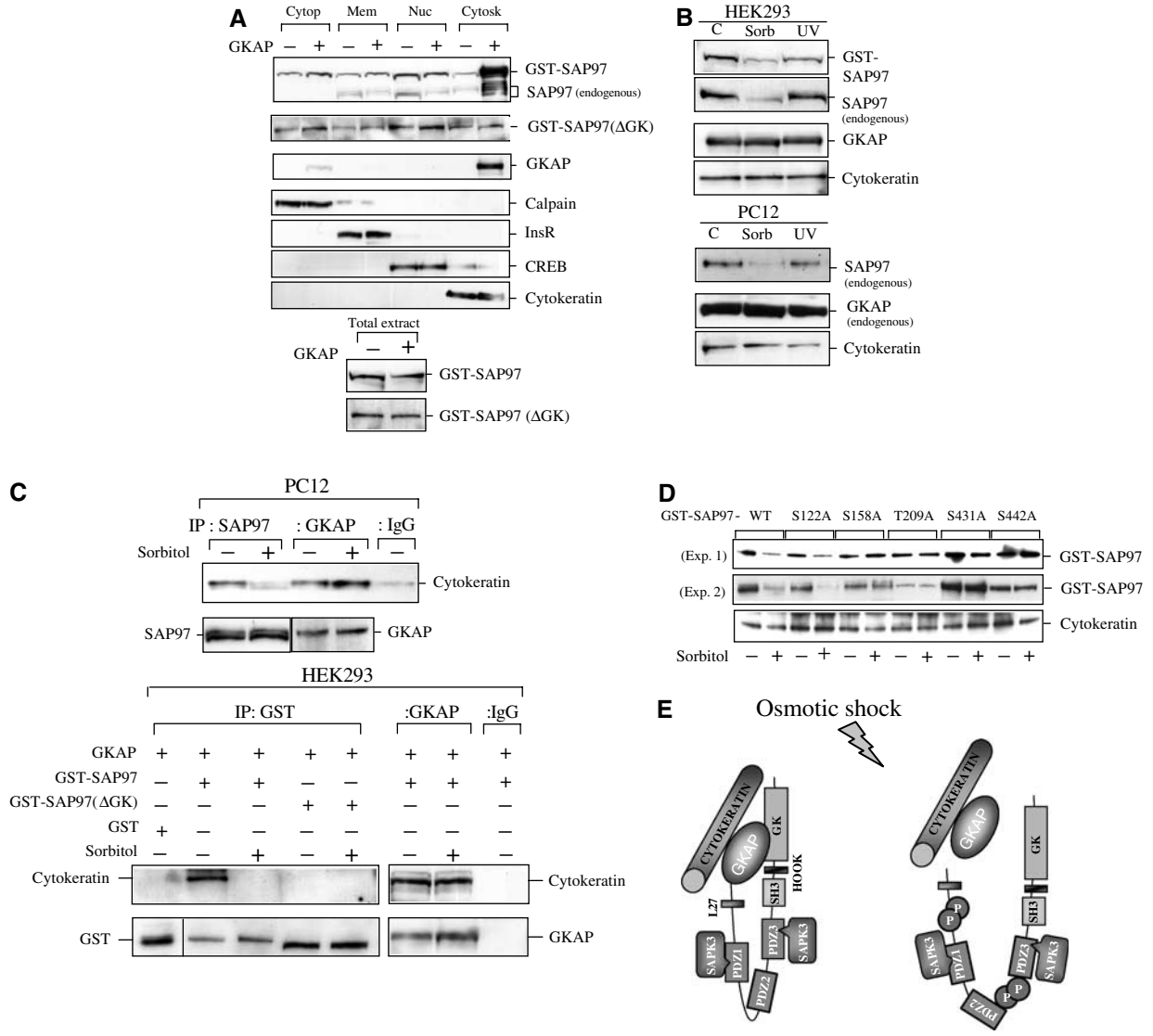


Figure 6 SAP97/hDlg is recruited to the cytoskeleton through GKAP. (**A**) HEK293 cells transfected with SAP97/hDlg either FL (GST-SAP97) or lacking the GK domain (GST-SAP97(ΔGK)) with or without GKAP were subjected to cellular fractionation, and 40 μg of protein from each cell fraction was immunoblotted using the antibodies indicated in the figure. (**B**) HEK293 cells co-transfected with SAP97/hDlg and GKAP or undifferentiated PC12 cells were left unstimulated or exposed for 15 min to 0.5 M sorbitol or to UV-C radiation (200 J/m^2), followed by a 30 min incubation, and subjected to cellular fractionation. Then, 40 μg of protein from the cytoskeleton was immunoblotted using the antibodies indicated in the figure. (**C**) Untransfected undifferentiated PC12 cells (upper panel) or HEK293 cells (lower panel) transfected with SAP97/hDlg either FL (GST-SAP97) or lacking the GK domain (GST-SAP97(ΔGK)) or GST with GKAP (as indicated in the figure) and then exposed for 15 min osmotic shock (0.5 M sorbitol). Samples were then immunoprecipitated using anti-SAP97 or anti-GKAP antibodies or IgG as control, and the pellets were blotted with anti-cytokeratin, anti-GKAP or anti-GST antibody. (**D**) HEK293 cells were transfected with either WT GST-SAP97 or different GST-SAP97 mutants, and then exposed for 15 min osmotic shock (0.5 M sorbitol). Cytoskeletal fraction was extracted and 40 μg of protein blotted using anti-SAP97 antibody or anti-cytokeratin antibody as loading control. Results from two different experiments (Exp. 1 and Exp. 2) are shown. Quantification of the protein detected in panels B, C and D are shown in Supplementary Figure 4. (**E**) GKAP-SAP97/hDlg complex is associated with the cytokeletal fraction under normal physiological conditions. Changes in the osmolarity of the environment activate SAPK3/p38γ, which phosphorylates SAP97/hDlg, causing a conformational change and its dissociation from GKAP and therefore from the cytoskeleton.

As expected, the amount of WT SAP97/hDlg interacting with cytoskeleton decreased following osmotic shock, but every SAP97/hDlg phosphorylation site mutant, except S122A in which phosphorylation is not regulated by hyperosmotic shock, remained bound to the cytoskeleton in either untreated or shocked cells (Figure 6D). All these results indicate that GKAP is located at the cytoskeleton probably forming a complex with other proteins, and recruits SAP97/hDlg to this compartment. Upon activation of SAPK3/p38γ and phosphorylation of SAP97/hDlg, this may suffer a conformational change that dissociated it from GKAP and the cytoskeletal fraction (Figure 6E).

Discussion

In this study, we identify SAP97/hDlg, a homologue of the *Drosophila* tumour suppressor dlg, as a new physiological substrate for SAPK3/p38γ. We also show that SAPK3/p38γ associates *in situ* with PDZ domains 1 and 3 of SAP97/hDlg and that this interaction occurs via the C-terminal sequence of the kinase. Moreover, SAPK3/p38γ binds to SAP97/hDlg in mouse brains (Supplementary Figure 3). SAP97/hDlg is not the only member of the MAGUK family that interacts with SAPK3/p38γ. Recently, we have shown that other member of this family, SAP90/PSD95, associates and becomes phosphorylated in cells in response to mitogens or cellular stresses, phosphorylation being mediated by ERK1/2 or SAPK3/p38γ, respectively (Sabio *et al*, 2004). This finding raised the possibility that phosphorylation in response to external stimuli may be a new mechanism for regulating the function of members of the MAGUK family.

Moreover, SAP97/hDlg becomes phosphorylated at four residues in cells after exposure to stresses, but not to mitogens (results not shown). These findings establish that SAP97/hDlg is a physiological substrate for one or more stress-activated kinases. The results obtained using protein kinase inhibitors in combination with the peptide TatSAPK3C, which specifically blocks the phosphorylation of PDZ-domain-containing proteins by SAPK3/p38γ (Sabio *et al*, 2004), strongly suggest that SAPK3/p38γ is the stress-activated kinase that phosphorylates SAP97/hDlg in cells.

To obtain more definitive information, we generated mice deficient in SAPK3/p38γ, SAPK4/p38δ or both kinases. The knockout of SAPK3/p38γ or SAPK4/p38δ or the both kinases did not affect the expression or the activation of other components of the family, compared to that in WT cells. However, in SAPK4/p38δ knockout cells an increase in the activation and activity of SAPK3/p38γ is observed. This is not due to an increase in the levels of SAPK3/p38γ expression in these cells. It could be explained by an up-regulation of one of its upstream activators, MKK3 or MKK6. If this is the case, then one function of SAPK4/p38δ may be to limit the activation of SAPK3/p38γ to ensure a correct cellular response to extracellular signals. It has been shown that p38α can directly control the concentration of its upstream activator MKK6 (Ambrosino *et al*, 2003). We are currently investigating if this is also the case in SAPK4/p38δ knockout cells. However, it is also possible that in the absence of SAPK4/p38δ, a greater proportion of the MKK3/MKK6 is diverted to the activation of SAPK3/p38γ.

We used primary mouse embryonic fibroblasts derived from the different knockouts and WT mice in combination with kinase inhibitors to elucidate the role of SAPK3/p38γ on the phosphorylation of SAP97/hDlg. Our results clearly show that, in WT MEF, endogenous SAP97/hDlg is phosphorylated at three residues in response to osmotic shock. We also provide evidence that this phosphorylation is mediated by SAPK3/p38γ, despite SAP97/hDlg also becomes phosphorylated in SAPK3/p38γ(−/−) cells due to the compensatory activity of other p38 MAPKs. Our results are a clear example of the functional redundancy caused by the existence of highly related family members. This issue is demonstrated here by the use of cells from mouse knockouts lacking multiple family members in combination with the use of specific inhibitors for the different kinases. Such redundancy may account for the failure on finding a phenotype in the SAPK3/p38γ(−/−) mice and point to the need to make a knock-in mouse expressing inactive SAPK3/p38γ.

This study also raises the question of the physiological role of the association of SAP97/hDlg with SAPK3/p38γ and of its phosphorylation by this kinase. One possibility we have examined is that the phosphorylation of SAP97/hDlg regulates its association with different binding partners. For example, it has been shown that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)-phosphorylation of SAP97/hDlg disrupts its interaction with the NMDA receptor subunit (NR2A) both *in vitro* and in transfected COS-7 cells (Gardoni *et al*, 2003). Using a mutational and transgenic expression approach in flies, it has been shown that dlg interacts with CaMKII and that its synaptic localisation is regulated through its phosphorylation by CaMKII (Koh *et al*, 1999). In addition, it has been suggested that the phosphorylation state of SAP97/hDlg also regulates its association to the p85/PI-3 kinase complex in human epithelial cells (Laprise *et al*, 2003).

This also appears to be the case for SAPK3/p38γ because we show here that interaction between SAP97/hDlg and the protein GKAP is regulated by the phosphorylation state of SAP97/hDlg *in vitro* and in cells. The protein GKAP, also known as SAPAP, seems to have a role in the molecular organisation of synapses and also in neuronal cell signalling by linking receptors to the cytoskeleton via its interaction with MAGUK family members, and the protein Shak/ProSAP that interacts with actin through Cortactin (Du *et al*, 1998; Boeckers *et al*, 1999; Naisbitt *et al*, 1999). Northern and Western blots of GKAP showed that their expression was almost restricted to brain (Kim *et al*, 1997; Takeuchi *et al*, 1997), although recently, it has been reported that GKAP is also expressed in muscle at the neuromuscular junction (Parker *et al*, 2004). Surprisingly, we detect the existence GKAP protein in HEK293 cells, although the level of expression is approx. 10-fold lower than in neuroblastoma, PC12 cell or brain (results not shown). Our results also indicate that all endogenous GKAP is associated with SAP97/Dlg in PC12 and HEK293 cells.

GKAP associates with intermediate filaments and neurofilaments (Naisbitt *et al*, 1999). In this study we show that GKAP is located at the cytoskeleton probably forming a complex with other proteins, and recruits SAP97/hDlg to this compartment. Upon activation of SAPK3/p38γ and phosphorylation of SAP97/hDlg after hyperosmotic shock, this protein is dissociated from GKAP and the cytoskeletal fraction. It has been shown utilising crystal structure data available for PDZ, SH3 and GK domains that SAP97/hDlg can exist

in a compact U-shaped conformation in which the N-terminal domain folds back and interact with the SH3 and GK domain, at the C-terminus of the molecule facilitating GKAP binding (Wu *et al*, 2000). Although, a more detailed analysis of the structural changes caused by phosphorylation of SAP97/hDlg are required, our results suggest that when this is fully phosphorylated, after osmotic shock, the structure at it N-terminal domain suffers a conformational change big enough to affect the association of GKAP to the GK domain of SAP97/hDlg. This is a novel regulatory pathway for the adaptation of cells to hyperosmolar environment that acts in parallel with the classical p38α pathway, which play a role on remodeling cytoskeletal proteins in other ways (Ito *et al*, 1997; Landry and Huot, 1999, Bustamante *et al*, 2003; Chang and Goldman, 2004). The pathway identify in this paper involves the protein kinase SAPK3/p38γ and its novel substrate SAP97/hDlg, and modulates the cytoskeletal protein composition by the phosphorylation of one of its components in response to hyperosmotic shock. This pathway could play a role in regulating various signalling events involved in cytoskeletal crosstalk (Chang and Goldman, 2004) and it could also be implicated in maintaining cellular polarity, cell size and the integrity of the intercellular-junctional complexes under conditions in which the osmolarity of the cellular environment change.

Materials and methods

Antibodies

To generate five different phospho-specific antibodies that recognise SAP97 phosphorylated at sites Ser^{122} (antibody Phos-Ser122), Ser^{158} (antibody Phos-Ser158), Thr^{209} (antibody Phos-Thr209), Ser^{431} (antibody Phos-Ser431) or Ser^{442} (antibody Phos-Ser442), the peptides (SERIpSPQVPN) (residues 118–127), VSHSHIpSPIK (residues 152–161), NTDSLEpTPTYVNG (residues 203–215), DNHVpSPSSYLGQTPASPARYSPISK and DNHVSPSSYLGQTPAp-SPARYSPISK (residues 427–451) of rat SAP97 were coupled to bovine serum albumin and keyhole limpet haemocyanin and injected into sheep at Diagnostics Scotland (Pennicuik, UK). All peptides used in this study were synthesised by Dr G Bloomberg (University of Bristol, UK). An antibody that recognises both phosphorylated and nonphosphorylated SAP97 was generated by injecting sheep with glutathione *S*-transferase (GST)-SAP97. The antisera were affinity purified as previously described (Sabio *et al*, 2004).

The anti-GKAP antibody was raised against the peptide SATESADSIEIYVPEAQTRL corresponding to the last 20 residues. The anti-PBK antibody was raised against the peptide DRPSAAHI-VEALETDV corresponding to residues (307–322) of PBK. The antibodies were affinity purified as described above. Anti-CASK antibody was obtained from Upstate Inc. (Dundee, UK). Other antibodies used in this study have been previously described (Kuma *et al*, 2004; Sabio *et al*, 2004).

Identification of the phosphorylation sites in SAP97

Rat GST-SAP97 (1 μM) was incubated for 1 h at 30°C with activated (0.5 U/ml) GST-SAPK3 or GST-SAPK4, 10 mM magnesium acetate and 100 μM [γ-^{32}P]ATP in a total volume of 50 μl containing 50 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1% (v/v) 2-mercaptoethanol. After SDS–PAGE, the band corresponding to ^{32}P-labeled SAP97 was excised, digested with trypsin and chromatographed on a Vydac 218TP54 C_{18} column equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The column was developed with a linear acetonitrile gradient. The major peaks of ^{32}P radioactivity were analysed by mass spectrometry and Edman sequencing to determine the peptide sequences and to identify the sites of phosphorylation as in Campbell and Morrice (2002). Peak 2 in Figure 1 required subdigestion with 0.1 μg Asp-N in 20 mM NH_4HCO_3 plus 0.1% NOG in order to identify the phosphorylated residue(s) in that peptide.

Cell culture, transfection and lysis

HEK293 cells, HeLa, PC12 cells and MEF were cultured as described previously (Sabio *et al*, 2004). SH-SY5Y human neuroblastoma cells were grown in DMEM-F12 supplemented with 15% (v/v) fetal bovine serum, 2 mM glutamine and 1% (v/v) nonessential amino acids. To differentiated SH-SY5, cells were cultured in the same medium containing 100 ng/ml NGF. Transfection of cells was carried out using FuGENE 6 (Roche), following the protocol recommended by the manufacturer or using the calcium phosphate method (Sabio *et al*, 2004). Cells were incubated in DMEM for 12 h in the absence of serum before exposure to 0.5 M sorbitol or UV-C radiation (200 J/m^2) as indicated in the figure legends, then lysed in buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 50 mM sodium β-glycerophosphate, 5 mM pyrophosphate, 0.27 M sucrose, 0.1 mM phenylmethylsulphonyl fluoride, 1% (v/v) Triton X-100) plus 0.1% (v/v) 2-mercaptoethanol and complete Proteinase inhibitor cocktail (Roche, East Sussex, UK). Lysates were centrifuged at 13 000 *g* for 15 min at 4°C, the supernatants removed, quick frozen in liquid nitrogen and stored at −80°C until use. Where required, cells were pre-incubated for 1 h with 10 μM SB203580 (Calbiochem, Nottingham) and/or 5 μM PD184352 (made by customer synthesis), or with 400 μM (final concentration) of peptides TatSAPK3C(WT) (YGRKKRRQRRRARVPKETAL) or TatSAPK3C(AA) (YGRKKRRQRR RARVPKAAAA) prior to stimulation with the above-mentioned agonists.

Immunoprecipitation from cell lysates

Extracts from HEK293 or SH-SY5Y cells (1–10 mg protein, see figure legends) were incubated with 4 μg SAP97 or 5 μg GKAP antibody coupled to protein G-Sepharose. After incubation for 2 h at 4°C, the captured proteins were centrifuged at 13 000 *g*, the supernatant discarded and the beads washed twice in buffer A containing 0.5 M NaCl, then twice in buffer A alone. In experiments where SAP97-associated proteins were studied, SAP97/hDlg was immunoprecipitated as above and the beads washed four times in buffer A containing 0.15 M NaCl. Samples were electrophoresed and then immunoblotted.

Immunofluorescence staining

It was performed as previously described (Sabio *et al*, 2004).

Subcellular fractionation

Subcellular fractionation was performed using the ProteoExtract™, Subcellular Proteome Extraction Kit from Calbiochem (Nottingham). Immunoblotting with the following antibodies against the indicated marker proteins were carried out as control: anti-calpain for cytosolic fraction, anti-Insulin receptor (InsR) for the membrane fraction, anti-CREB for the nuclear and anti-pan-cytokeratin for the cytoskeletal fraction.

Targeted disruption of SAPK3/p38γ or SAPK4/p38δ gene

The *SAPK3/p38γ* or *SAPK4/p38δ* gene was targeted in ES cells using a targeting vector designed to delete exon 3 of the *SAPK3/p38γ* gene or from the second half of exon 4 to the middle of exon 7 of the *SAPK4/p38δ* gene and replace them with a neomycin-resistant gene. The targeting vectors consisted of approx. 8 kb genomic sequences (2 kb in the 5′ and 6 kb in the 3′ arm), a PGK-driven neomycin resistance marker and thymidine kinase for negative selection (Figure 3). Electroporated ES cells were cultured as described (Wiggin *et al*, 2002), selected using G418 and gancyclovir, and screened by both PCR analysis and Southern hybridisation according to standard protocols. Targeted ES cells were injected into C57BI/6J × BALB/c blastocysts, and the resulting chimaeric males were successfully used to establish a line. Genotypes were determined by PCR screening of tail biopsies. PCR primers used for SAPK3/p38γ knockout mice were CCTGAGGTTTAGATAGGCTG TATGTCTCACTCACAC (targeted or WT P1), CACTTCGCCCAATAG CAGCCAGTCCCTTCC (targeted P2) and GAATTCCCAGTAGGTCA TTCCTGGGACCATCC (WT P3). PCR primers used for SAK4/p38δ knockout mice were CCCTTGAGCCATAGATCCTGGACTTTGG (WT P4), CATGAGCTTGAGATGCTCTCTGGGACAC (targeted or WT P5) and GGCGATGCCTGCTTGCCGAATATCATGG (targeted P6).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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