Stress- and mitogen-induced phosphorylation of the synapse-associated protein SAP90/PSD-95 by activation of SAPK3/p38 γ and ERK1/ERK2

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SAPK3 (stress-activated protein kinase-3, also known as p38 γ) is a member of the mitogen-activated protein kinase family; it phosphorylates substrates in response to cellular stress, and has been shown to bind through its C-terminal sequence to the PDZ domain of α 1-syntrophin. In the present study, we show that SAP90 [(synapse-associated protein 90; also known as PSD-95 (post-synaptic density-95)) is a novel physiological substrate for both SAPK3/p38 γ and the ERK (extracellular-signal-regulated protein kinase). SAPK3/p38 γ binds preferentially to the third PDZ domain of SAP90 and phosphorylates residues Thr²⁸⁷ and Ser²⁹⁰ *in vitro*, and Ser²⁹⁰ in cells in response to cellular stresses. Phosphorylation of SAP90 is dependent on the binding of SAPK3/p38 γ to the PDZ domain of SAP90. It is not blocked by SB 203580, which inhibits SAPK2a/p38 α and SAPK2b/p38 β but not SAPK3/p38 γ , or by the ERK pathway inhibitor PD 184352. However, phosphorylation is abolished when cells are treated with a cell-permeant Tat fusion peptide that disrupts the interaction of SAPK3/p38 γ with SAP90. ERK2 also phosphorylates SAP90 at

Thr²⁸⁷ and Ser²⁹⁰ *in vitro*, but this does not require PDZ-dependent binding. SAP90 also becomes phosphorylated in response to mitogens, and this phosphorylation is prevented by pretreatment of the cells with PD 184352, but not with SB 203580. In neurons, SAP90 and SAPK3/p38 γ co-localize and they are co-immunoprecipitated from brain synaptic junctional preparations. These results demonstrate that SAP90 is a novel binding partner for SAPK3/p38 γ , a first physiological substrate described for SAPK3/p38 γ and a novel substrate for ERK1/ERK2, and that phosphorylation of SAP90 may play a role in regulating protein–protein interactions at the synapse in response to adverse stress- or mitogen-related stimuli.

Key words: extracellular-signal-regulated kinase (ERK), phosphorylation, stress, stress-activated protein kinase-3 (SAPK3)/p38 γ , synapse, synapse-associated protein 90 (SAP90)/post-synaptic density-95 (PSD-95).

INTRODUCTION

SAPKs (stress-activated protein kinases) are MAPK (mitogen-activated protein kinase) family members, which are activated by cellular stresses, bacterial lipopolysaccharides and cytokines [1]. A major challenge in this field is to identify the physiological substrates and functions of each SAPK. The group of SAPK/p38 comprises SAPK2a/p38 α , SAPK2b/p38 β , SAPK3/p38 γ [also known as ERK6 (extracellular-signal-regulated kinase 6)] and SAPK4/p38 δ . Identification of physiological substrates for SAPK2a/p38 α and SAPK2b/p38 β has been greatly facilitated by the availability of specific inhibitors of these enzymes, such as the cell-permeant pyridyl imidazole SB 203580 and related compounds [2]. Substrates for SAPK2a/p38 α and SAPK2b/p38 β include other protein kinases, as well as several transcription factors [3]. SAPK3/p38 γ and SAPK4/p38 δ are not inhibited by SB 203580 [4,5], and consequently very little information is available about their substrates. Stathmin and the elongation factor 2 kinase have been proposed as physiological substrates for SAPK4/p38 δ [6–8], whereas the activating transcription factor-2, microtubule-as-

sociated protein Tau and the protein α 1-syntrophin are substrates of SAPK3/p38 γ *in vitro* [9–12].

Phosphorylation of α 1-syntrophin by SAPK3/p38 γ depends on the interaction of the C-terminal sequence-ETXL of the kinase with the PDZ domain of α 1-syntrophin. In skeletal muscles, SAPK3/p38 γ and α 1-syntrophin were found to co-localize at the neuromuscular junction and throughout the sarcolemma [11]. PDZ domains are modular protein–protein interaction domains that serve to localize proteins to specific subcellular sites. Many proteins with PDZ domains are localized to specialized sites of cell–cell contact, such as synapses and tight junctions, where they bind to the C-termini of transmembrane proteins and other structural proteins, thereby creating a mechanism for positioning and clustering these proteins and for connecting them to the cytoskeletal network. The finding that SAPK3/p38 γ binds through its C-terminal sequence to the PDZ domain of α 1-syntrophin and that the phosphorylation of α 1-syntrophin by SAPK3/p38 γ depends on this interaction, enabled us to identify a novel mechanism for targeting a protein kinase to its membrane-associated substrate [11]. Protein phosphorylation may also be

Abbreviations used: DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; GFP, green fluorescent protein; GK, guanylate kinase; GST, glutathione S-transferase; HEK-293 cells, human embryonic kidney-293 cells; Hsp27, heat-shock protein 27; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKAP, MAP kinase-activated protein; MBP, myelin basic protein; NGF, nerve growth factor; PICK, proteins that interact with protein C-kinase; PKC, protein kinase C; PSD, postsynaptic density; SAP, synapse-associated proteins; SAPK, stress-activated protein kinase; SH3, src homology 3.

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important for modulating the interactions between PDZ domain-containing proteins and their binding partners.

Although SAPK3/p38 γ is most significantly expressed in skeletal muscles, it is also expressed in many other tissues including the brain [9,13,14]. Thus it is probable that SAPK3/p38 γ will be found to interact with PDZ domains of proteins other than α 1-syntrophin. Possible candidates include members of the class I PDZ domains, such as the SAP90 [(synapse-associated protein 90; PSD-95 (postsynaptic density-95)] and its homologues SAP97, SAP102 and PSD-93/Chapyn110, which recognize the motif S/T-X-Hyd-CO₂H, similar to the C-terminus of SAPK3/p38 γ [15,16]. Members of the SAP90 family are localized at synapses and can be found both pre- and postsynaptically. In particular, SAP90 is enriched in the PSD associated with membranes of excitatory synapses. PSD consists of a high concentration of glutamate receptors that are integrated into a complex network of regulatory, scaffolding, and cytoskeletal proteins [17] and functions as a postsynaptic organelle specialized for glutamatergic signal transduction.

All SAP90 family members have a similar structural organization comprising three N-terminal PDZ domains, followed by an SH3 (src homology 3) domain and a GK (guanylate kinase)-like region [18,19]. Functionally, SAP90 family members are thought to function as structural scaffolding proteins that assemble multicomponent protein complexes facilitating signal transduction. For example, NR2 subunits of the N-methyl-D-aspartate receptor have been found to bind the first two PDZ domains in SAP90 [20], and disrupting this interaction interferes with N-methyl-D-aspartate receptor-mediated synaptic plasticity [21]. SAP90, via other domains, is also capable of interacting with cytoskeletal proteins and adhesion molecules such as CRIPT (cysteine-rich interactor of PDZ three), GKAP (GK-associated protein)/SAPAP (SAP 90/PSD-95-associated protein), Citron and neurologin, as well as proteins involved in signal transduction including neuronal nitric oxide synthase, and Ras and Rho GTPase-activating proteins, Syn GTPase-activating protein and spine-associated RapGAP [18,19,22–24]. Recent studies have also shown that the SH3–GK domains in SAP90 will bind AKAP79 (A-kinase anchoring protein-79), which is supposed to direct the phosphorylation by protein kinase A of specific PSD protein, such as subunits of the glutamate receptors [25].

An important unresolved issue, with regard to the ability of SAP90 family members to build multicomponent protein complexes, is which kinases and phosphatases act directly on SAP90 family members regulating their association with different binding partners. In the present study, we have found that the SAPK, SAPK3/p38 γ not only binds directly to the third PDZ domain in SAP90, but also phosphorylates specific serine and threonine residues located in the N-terminal half of this synaptic protein. SAP90 was also found to be a physiological substrate for ERK1/ERK2. Importantly, we have found that SAPK3/p38 γ and SAP90 co-localize at synapses and interact in synaptic junctional preparations. These findings indicate that SAPK3/p38 γ -dependent binding and phosphorylation of SAP90 might regulate its association with synaptic proteins in response to stress-related signalling events.

MATERIALS AND METHODS

Materials

Precast polyacrylamide gels, running buffer and transfer buffer were from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.). SB 203580 was obtained from Calbiochem (Nottingham, U.K.) and MBP (myelin basic protein) from Gibco BRL (Paisley). Complete

proteinase inhibitor cocktail tablets were from Roche (Lewes, East Sussex, U.K.). All peptides were synthesized by Dr G. Bloomberg (University of Bristol, U.K.). PD 184352 was prepared by custom synthesis. Other chemicals were of the highest purity available and purchased from Merck (Poole, Dorset, U.K.) or Sigma–Aldrich (Poole).

Antibodies

The rabbit SAP90 polyclonal antibody (5'2d) was generated against the first 63 N-terminal residues of SAP90 [26]. The 9E10 monoclonal antibody recognizes the c-myc epitope. The peptides YPTAMphosTPTSPR (residues 282–292) and AMTPTphos-SPRRYS (residues 285–295) of SAP90 were coupled with BSA and keyhole limpet haemocyanin and injected into sheep at Diagnostos Scotland (Pennicuik, U.K.). The antisera were affinity-purified on a phospho-peptide antigen-Sepharose column, and then passed through another column to which the unphosphorylated form of the peptide had been bound. The flow-through fractions were collected and used at 1 μ g/ml for immunoblotting. An antibody that recognizes both phosphorylated and non-phosphorylated SAP90 was generated by injecting sheep with GST (glutathione S-transferase)-tagged SAP90. The antibody was affinity-purified on GST–Sepharose to remove anti-GST antibodies and then on GST–SAP90–Sepharose. An antibody [anti-PSD-95(clone K28/43)] that immunoprecipitates and recognizes SAP90 in immunofluorescence staining was purchased from Upstate (Dundee, U.K.).

Anti-SAPK3 antibodies were raised and purified as described in [9] and used for immunoblotting. An antibody that recognizes SAPK3/p38 γ in immunofluorescence staining was generated by injecting sheep with peptides corresponding to the N- and C-terminal regions of SAPK3/p38 γ . The antibody was affinity-purified on a GST–SAPK3 antigen-Sepharose column.

An antibody that recognizes the Hsp27 (heat-shock protein 27; phosphorylated at Ser¹⁵) was described previously [7]. Antibodies that recognize SAPK2a/p38 α phosphorylated at Thr¹⁸⁰ and Tyr¹⁸² (these antibodies also recognize phosphorylated SAPK2b/p38 β , SAPK3/p38 γ and SAPK4/p38 δ), ERK1/ERK2, phospho-ERK1/ERK2 (Thr²⁰²/Tyr²⁰⁴) and phospho-MAPKAP-K2 (where MAPKAP stands for MAP kinase-activated protein; Thr³³⁴) were purchased from New England Biolabs (Hitchin, Herts., U.K.) and a SAPK2a/p38 α antibody was obtained from Upstate. An antibody that recognizes GFP (green fluorescent protein), and secondary antibodies Alexa-Fluor 488-conjugated donkey anti-mouse and Alexa-Fluor 594-conjugated donkey anti-sheep were obtained from Molecular Probes (Leiden, The Netherlands). Rabbit anti-sheep IgG, goat anti-rabbit and rabbit anti-mouse IgG antibodies conjugated to peroxidase and peroxidase-conjugated Protein G were obtained from Perbio Science (Tattenhall, U.K.).

DNA constructs and protein expression

Protein kinases used in the present study, GST–JNK3 (c-Jun N-terminal kinase; lacking the first 39 amino acids) and GST–SAP90, were expressed in *Escherichia coli* strain BL21 and purified as described in [7]. For overexpression, GST-tagged SAP90 was in the vector pEBG-2T, whereas GFP–SAPK3 full-length (FL)(1–367) and GFP–SAPK3(Δ C)(1–364) were in the expression vector EGFP–C3.

Overlay filter binding assay

Full-length rat GST–SAP90 (residues 1–724) was subcloned into pGEX2T (Amersham Biosciences, Uppsala, Sweden). GST-fusion constructs encoding PDZ domain 1 (residues 53–153),

domain 2 (residues 151–253), domain 3 (residues 301–401) and the SH3-GK domain (residues 412–724) of SAP90 were subcloned into pGEX4T-2 (Amersham Biosciences). Full-length rat GST-SAPK3 (residues 1–367) was subcloned into pET32a (thioredoxin fusion vector; Novagen, Nottingham, U.K.). GST-fusion proteins were purified using glutathione-Sepharose 4B (Amersham Biosciences) and thioredoxin proteins were purified using His-Bind metal chelation resin (Novagen). For the overlay filter binding assay, 250 ng of each purified target protein was run on SDS/polyacrylamide gel, transferred on to a PVDF membrane and incubated overnight with 5 % milk/PBS, followed by a 1 h incubation at room temperature (20 °C) with 10 μ g/ml proving protein (thioredoxin-SAPK3 or thioredoxin) in 0.1 % Tween 20/2 % milk. After washing with 0.1 % Tween 20/PBS, the membrane was incubated for 1 h at room temperature with anti-thioredoxin antibody (1:5000; Invitrogen) in 2 % milk/PBS. After a further wash, it was incubated for 1 h with biotinylated secondary antibody (1:500; Vector Laboratories, Burlingame, CA, U.S.A.) in 10 % (v/v) foetal calf serum/PBS and washed. This was followed by a 30 min incubation with avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) in PBS and a further wash. The signal was developed using diaminobenzidine/NiCl₂. GST-fusion proteins were visualized using an anti-GST antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

Cell culture, transfection and lysis

PC12 cells were cultured in RPMI 1640 and HEK-293 cells (human embryonic kidney-293 cells) in DMEM (Dulbecco's modified Eagle's medium) at 37 °C, supplemented with 10 % foetal calf serum, 50 units/ml penicillin, 50 μ g/ml streptomycin (Life Technologies, Paisley, Renfrewshire, Scotland, U.K.) and 2 mM glutamine (BioWhittaker, Wokingham, Berks., U.K.). HEK-293 cells were transfected using FuGENE 6 (Roche) according to the manufacturer's instructions. After splitting cells to a density of 1×10^6 /6 cm dish, and incubating for 24 h at 37 °C in an atmosphere of 95 % O₂/5 % CO₂, 1 μ g DNA (previously incubated for 15 min at room temperature with 6 μ l of FuGENE 6 reagent in 100 μ l of DMEM) was added. After 36 h, cells were stimulated with the agonists as indicated below. They were incubated in DMEM for 12 h in the absence of serum before stimulation.

Cells were exposed to 0.5 M sorbitol, UV-C radiation (200 J/m²), 100 ng/ml EGF (epidermal growth factor) or 400 ng/ml PMA 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 PP_i/0.27 M sucrose/0.1 mM PMSF/1 % (v/v) Triton X-100] plus 0.1 % (v/v) 2-mercaptoethanol and complete proteinase inhibitor cocktail. Lysates were centrifuged at 13 000 *g* for 15 min at 4 °C, the supernatants were removed, quick-frozen in liquid nitrogen and stored at –80 °C until use. Where required, cells were preincubated for 1 h with 10 μ M SB 203580 and/or 5 μ M PD 184352, or with 400 μ M (final concentration) of TatSAPK3C peptide (YGRKKRRQRRRARVPK-ETAL) or dansyl-TatSAPK3C before stimulation with the above-mentioned agonists.

Extracts from PC12 cells (5 mg of protein) were incubated with 4 μ g of SAP90 antibody coupled with protein G-Sepharose. After incubation for 2 h at 4 °C, the captured proteins were centrifuged at 13 000 *g*, the supernatant was discarded and the beads washed twice in buffer A containing 0.5 M NaCl, then twice in buffer A alone. Samples were denatured, electrophoresed and then immunoblotted.

Primary cultures of cerebellar granule cells were obtained from 5-day-old mouse brain, as described in [27]. After 7 days in culture, the cells were used for immunofluorescence staining as described below. PC12 cells were differentiated in serum-free medium in the presence of 100 ng/ml NGF (nerve growth factor) for 4 days before being used for immunofluorescence staining experiments.

Primary cultures of hippocampal neurons were prepared from embryonic day 19 hippocampi and grown for 21 DIV (days *in vitro*) as described in [19].

Immunofluorescence staining

PC12 cells were grown on type IV collagen and cerebellar granular cells on polylysine-coated 22 mm $\times$ 22 mm glass coverslips for 24 h before fixation. Coverslips were washed twice with PBS and fixed with 4 % (w/v) paraformaldehyde in PBS for 10 min. Cells were rinsed five times in PBS, incubated for 15 min in PBS containing 50 mM ammonium chloride followed by permeabilization for 1 min with PBS containing 1 % Triton X-100. Non-specific staining was blocked by incubating the coverslips in PBS containing 5 % (w/v) BSA, 2 % (w/v) donkey serum and 50 mM ammonium chloride for 1 h in a humidified chamber. SAPK3 and SAP90 antibodies were diluted in PBS containing 5 % BSA, and incubation with the antibodies was performed simultaneously for 2 h at room temperature in a humidified chamber. SAPK3/p38 γ and SAP90 were immunostained by using 12 μ g/ml SAPK3 antibody or 4 μ g/ml SAP90 antibody respectively. Secondary antibodies were applied for another hour in a dark humidified chamber and used at 1:250. Fixation, permeabilization and immunostaining of the cells were performed at room temperature. Hippocampal neurons were grown for 21 DIV, fixed with methanol and stained with SAP90 and SAPK3 antibodies as described in [28]. Epifluorescence microscopy was performed using a Leica DM-IRS or Zeiss axiovert 200 M inverted microscope equipped with PlanApo $\times$ 63 1.32NA oil immersion objectives. Images were captured using Improvision® Openlab 3.0.7 software and assembled using Adobe Photoshop® 4.0.

Preparation of brain fractions

Rat brain fractions were prepared from 30-day-old Sprague-Dawley rat essentially as described in [29]. Briefly, brains were dissected, washed and homogenized in 0.3 M sucrose in 2 mM Hepes and centrifuged at 6000 *g* for 10 min. The supernatant was then centrifuged at 17 000 *g* for 20 min. The pelleted material was resuspended in 5 ml of 0.3 M sucrose in 2 mM Hepes and carefully pipetted on to a sucrose gradient (0.8, 1.0 and 1.2 M sucrose). This sample was centrifuged at 85 000 *g* for 2 h (Beckmann ultracentrifuge, SW 41Ti). The four separated phases (P2A, myelin; P2B, endoplasmic reticulum/Golgi/plasma membranes; P2C, total synaptosomes; and P2D, mitochondria) were isolated and processed for Western blotting or immunoprecipitation. P2C was further separated into subcellular fractions by dilution in 4 vol. of 0.3 M sucrose in 2 mM Hepes, followed by centrifugation at 30 000 *g* for 25 min. The pellet was resuspended in 3.5 ml of lysis buffer (2 mM Hepes/0.5 mM EGTA) and slowly rotated at 4 °C for 45 min. This suspension was subsequently centrifuged at 10 000 *g* for 25 min. The resulting supernatant represents the soluble synaptosome fraction and the pellet synaptic plasma membranes.

Assay of SAPK3/p38 γ and other MAPK family members

SAPK3/p38 γ and other MAPK family members were assayed routinely using MBP as substrate [9]. Phosphorylation of SAP90

Figure 1 Interaction of SAPK3/p38 γ with SAP90

(A) Interaction of SAPK3/p38 γ with SAP90/PSD-95. An overlay binding assay was used to assess the interaction between SAPK3/p38 γ and full-length SAP90, PDZ domains 1–3 of SAP90 and its SH3-GK domain. Results are expressed semi-quantitatively, ranging from strong (+++) to no (–) binding of SAPK3/p38 γ . (B) Co-immunoprecipitation of SAP90 and SAPK3/p38 γ in HEK-293 cells. HEK-293 cells were either doubly (a) or singly transfected with SAP90-GFP (a, b) or SAPK3 (a, c) and immunoprecipitated with antibodies against the N-terminus of SAP90 (5'2d), SAPK3 or the c-myc epitope (9E10). Western blots were probed either with SAP90 or SAPK3 antibodies. (C) Co-immunoprecipitation of SAPK3/p38 γ with SAP90. HEK-293 cells were co-transfected with GST-SAP90 and GFP-SAPK3 (FL, full length: 1–367) or GFP-SAPK3 (Δ C, lacking the last four amino acids: 1–363). GST-SAP90 from the lysates was bound to GSH-Sephacrose beads. The pellets were immunoblotted with anti-SAPK3 or anti-SAP90 antibodies as indicated in the Figure. GFP-SAPK3 was immunoprecipitated from the lysates using an anti-GFP antibody. The immunoprecipitates were immunoblotted with anti-SAP90 or anti-SAPK3 antibodies.

by wild-type GST-SAPK3(1–367) and GST-SAPK3(1–363) was performed similarly. Reactions were stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid and, after centrifugation at 13 000 g for 10 min, the supernatants were discarded. The pellets were washed three times with 1 ml of 25% trichloroacetic acid and 32 P incorporation measured by Cerenkov counting. Incorporation of phosphate into substrate was kept below 0.2 mol of phosphate/mol of substrate, to ensure that initial conditions were met. One unit of activity was the amount that catalysed the incorporation of 1 nmol of phosphate into MBP in 1 min.

Identification of phosphorylation sites in SAP90

GST-SAP90 (1 μ M) was incubated for 1 h at 30 °C with activated GST-SAPK3 (2.0 units/ml), 10 mM magnesium acetate and 100 μ M [γ - 32 P]ATP in a total volume of 200 μ l of 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 0.1 mM sodium orthovanadate and 0.1% 2-mercaptoethanol. After SDS/PAGE and autoradiography, the band corresponding to 32 P-labelled SAP90 was excised, digested with chymotrypsin and chromatographed on a Vydac 218TP54 C₁₈ column equilibrated with 10 mM ammonium acet-

Figure 2 Expression and interaction of SAP90 and SAPK3 in subcellular fractions of adult rat brain

(A) Western blots of rat brain fractions, probed with antibodies against SAP90 (5'2d) or SAPK3, demonstrating the overlapping distribution of both proteins. Rbs, rat brain supernatant; rbp, rat brain pellet; P2C, synaptosomal membranes; SS, supernatant from lysed synaptosomes; SP, pellet of lysed synaptosomes; P2B, endoplasmic reticulum/Golgi/plasma membrane. (B) Interaction of SAP90 and SAPK3 in neuronal compartments. Western blots of different rat brain fractions, immunoprecipitated with the rabbit SAP90 5'2d antibody, the rabbit SAPK3 antibody and a mouse monoclonal antibody against the c-myc epitope (9E10) as control. Immunoblots were labelled with rabbit antibody against SAPK3/p38 γ and mouse antibody against SAP90. SAP90 and SAPK3 co-immunoprecipitate in synaptic plasma membrane fractions, but not in endoplasmic reticulum, Golgi or soluble fractions.

ate (pH 6.5), and the column was developed with a linear acetonitrile gradient. The flow rate was 0.8 ml/min and fractions of 0.4 ml were collected. The two major peaks of 32 P radioactivity were analysed by gas-phase sequencing [30] and electrospray ionization–mass spectrometry to determine the peptide sequences and to identify the sites of phosphorylation.

RESULTS

SAPK3/p38 γ interacts with SAP90

We have shown previously that the C-terminus of SAPK3/p38 γ binds to the PDZ domain of the protein α 1-syntrophin [11]. Since SAP90 is the prototype of a PDZ domain-containing protein, we investigated whether SAPK3/p38 γ interacts with SAP90 *in vitro*. An overlay filter-binding assay was used to examine the domains of SAP90 that interact with SAPK3/p38 γ (Figure 1A). Full-length SAP90 bound strongly to SAPK3/p38 γ , as did PDZ domain 3. PDZ domain 2 of SAP90 interacted more weakly, whereas PDZ domain 1 and the C-terminal SH3-GK domain failed to show a specific interaction with SAPK3/p38 γ . These results suggest that as with α 1-syntrophin [11], SAP90 binds SAPK3/p38 γ through one of its PDZ domains.

To assess whether SAPK3/p38 γ could bind SAP90 as a full-length protein in a cellular environment, we transfected HEK-293 cells with SAPK3/p38 γ and SAP90 either alone or together. Cell lysates were subjected to immunoprecipitation with antibodies against SAPK3/p38 γ , SAP90 or antibodies against a c-myc epitope (9E10) and Western-blotted (Figure 1B). In these experiments, antibodies to SAPK3/p38 γ were not only able to immunoprecipitate SAPK3/p38 γ , but, when present, also SAP90. Similarly, antibodies against SAP90 precipitated SAPK3/p38 γ as

Figure 3 Distribution of SAPK3/p38 γ and SAP90 in cells

Cerebellar granular cells (**A–C**), differentiated PC12 cells (**D–F**) and rat hippocampal neurons (**G–I**) were stained with anti-SAPK3 and Alexa Fluor 594-labelled secondary antibody (**A, D, G**) and with anti-SAP90 and Alexa Fluor 488-labelled secondary (**B, E, H**) antibody and were subjected to fluorescence microscopy. SAPK3/p38 γ and SAP90 signals are shown in red (**A, D, G**) and in green (**B, E, H**) respectively. In merged images (**C, F, I**), co-localized signals are shown in yellow. Scale bar, 10 μ m.

well as SAP90 when both proteins were co-transfected. Neither protein was immunoprecipitated with the 9E10 antibodies.

To assess the importance of the C-terminus of SAPK3/p38 γ in the binding to SAP90, we performed co-immunoprecipitations

of these proteins from HEK-293 cells transfected with SAP90 and either SAPK3 (FL, full length: 1–367) or SAPK3 (Δ C, 1–363: lacking the last four amino acids). As predicted, SAP90 only co-immunoprecipitated full-length SAPK3/p38 γ , but not

SAPK3/p38 γ with the C-terminal truncation SAPK3(Δ C) (Figure 1C). These results indicate that the last four amino acids of SAPK3/p38 γ , which conform to a type-1 PDZ-binding motif [11], are essential for its association with the PDZ domain of SAP90.

The strong interaction between SAP90 and SAPK3/p38 γ suggested that this interaction might be relevant *in vivo*. To test this hypothesis, we initially examined whether SAPK3/p38 γ is expressed in brain tissue and whether it co-fractionates with SAP90 in synaptic junctional preparations. This was accomplished by probing Western blots of rat brain fractions with antibodies against SAP90 and SAPK3/p38 γ . These results showed that both proteins are present in soluble and membrane-bound fractions, and are notably enriched in synaptosomal membranes, as well as in synaptic plasma membrane, but not in soluble synaptic fractions (Figure 2A). To assess whether SAP90 and SAPK3/p38 γ interact within these membrane compartments, we performed immunoprecipitations of each fraction with antibodies against SAPK3/p38 γ or SAP90 (5/2d). SAPK3/p38 γ and SAP90 could be co-immunoprecipitated from membrane-bound total brain homogenates, synaptosomal membrane and synaptic plasma membranes, but not from soluble fractions (Figure 2B). These findings suggest that SAP90 and SAPK3/p38 γ may interact with each other while associated with post-synaptic plasma membranes and not necessarily while in the cytosol.

SAP90 co-localizes with SAPK3/p38 γ in neurons

The ability of SAP90 and SAPK3/p38 γ to interact in synaptosomal and synaptic plasma membrane preparations, but not cytosolic fractions, indicates that the relevant interactions between these proteins occur at synapses. To confirm this, we compared the spatial distributions of SAP90 and SAPK3/p38 γ in cultured cerebellar and hippocampal neurons, as well as in NGF-treated PC12 cells, a tumour cell line that differentiates from immature chromaffin cells to a sympathetic neuron-like phenotype in response to NGF [31].

In differentiated PC12 cells, SAPK3/p38 γ immunoreactivity was found in the nucleus, cytoplasm and along neuritic processes. Similarly, in cerebellar granule cells, SAPK3/p38 γ was located in the nucleus, cytoplasm and along neuritic processes (Figures 3A and 3D). In both cell types, SAP90 exhibited a similar overlapping punctate distribution pattern in the cytoplasm and along neuritic processes (Figures 3B and 3E).

As synapses are not made by PC12 cells and are infrequent in cerebellar granule cell cultures, we compared the spatial distribution of SAPK3/p38 γ and SAP90 in hippocampal neurons cultured for 21 DIV. In these cells, SAP90 exhibits a punctate pattern along dendritic profiles (Figure 3H) that is typical of a postsynaptic distribution. At this stage, little immunoreactivity is seen in the cell soma. SAPK3/p38 γ immunoreactivity exhibited two patterns (Figure 3G). The first was a somato-dendritic distribution similar to that seen in PC12 cells and immature cerebellar neurons. The second was a punctate pattern along dendritic profiles that co-localized with SAP90 punctate. The latter is compatible with a synaptic localization of SAPK3/p38 γ , whereas the former is consistent with the presence of SAPK3/p38 γ in the cytosol. It should be noted that, whereas SAPK3/p38 γ and SAP90 were found in both cytosolic and membrane fractions, they were only found in a complex in synaptosomal and synaptic plasma membranes.

With regard to the specificity of the SAPK3/p38 γ staining, the patterns observed were abolished by incubation of the SAPK3 antiserum with recombinant SAPK3/p38 γ or with the N- and

Table 1 Comparison of substrate specificities of different MAPK family members

Each enzyme [at 0.5 unit/ml (a) or 0.1 unit/ml (b)] was assayed under initial rate conditions as described in the Materials and methods section.

(a)	
[Kinase] (0.5 unit/ml)	Rate of phosphorylation of 1 μ M SAP90 (%) relative to that of 1 μ M MBP (= 100 %)
SAPK3/p38 γ	100 $\pm$ 10
SAPK4/p38 δ	63 $\pm$ 4
MAPK2/ERK2	48 $\pm$ 7
SAPK2a/p38 α	2 $\pm$ 1
SAPK2b/p38 β	5 $\pm$ 1
(b)	
[Kinase] (0.1 unit/ml)	Rate of phosphorylation of 1 μ M SAP90 (%) relative to that of 1 μ M ATF2 (= 100 %)
SAPK3/p38 γ	100 $\pm$ 5
JNK2 α	6.2 $\pm$ 1
JNK3	4.0 $\pm$ 0.5

C-terminal peptides (results not shown). Furthermore, the SAPK3/p38 γ localization was confirmed with three different antibodies (results not shown).

SAP90 is phosphorylated *in vitro* by SAPK3/p38 γ and ERK2

SAP90 contains six (S/T-P) potential sites of phosphorylation by MAPK family members located outside of the PDZ domains. Three of these sites are located in the N-terminal region of the protein and the other three are found just before the PDZ domain 3. Initial rates of phosphorylation showed that SAP90 was a good substrate for SAPK3/p38 γ (Table 1). ERK2 and SAPK4/p38 δ phosphorylated SAP90 at lower rates, whereas SAPK2a/p38 α , SAPK2b/p38 β and JNK phosphorylated it weakly (Table 1).

At higher concentrations, SAPK3/p38 γ (2.0 units/ml) phosphorylated SAP90 *in vitro* to 2.0 mol of phosphate/mol of protein (results not shown). Phospho-amino acid analysis was used to show that phosphorylation occurred on serine and threonine residues (inner lanes in Figure 4). SAP90 phosphorylated by SAPK3/p38 γ was then digested with chymotrypsin and the resulting peptides were chromatographed on a C₁₈ column. Two major peaks of ³²P radioactivity, termed P1 and P2, were observed (Figure 4). P1 contained a peptide corresponding to residues 278–295 of SAP90. P2 contained two peptides, corresponding to residues 278–293 and 278–299. All three peptides were di-phosphorylated at Thr²⁸⁷ and Ser²⁹⁰. Residues Thr²⁸⁷ and Ser²⁹⁰ are located between PDZ domains 2 and 3 of SAP90.

The sites phosphorylated in SAP90 by ERK2 (Figure 4) and SAPK4/p38 δ (results not shown) were also analysed. P1 and P2 were the major peaks and both contained residues Thr²⁸⁷ and Ser²⁹⁰. P0 contained a peptide corresponding to residues 13–34, which was mono-phosphorylated at Ser²⁵ (Figure 4).

Interestingly, the phosphorylation of SAP90 by SAPK3/p38 γ *in vitro* was dependent on the extreme C-terminus of SAPK3/p38 γ . This was demonstrated by the following observations. First, SAPK3(1–363), a form of SAPK3/p38 γ that lacks the four C-terminal amino acids, phosphorylated SAP90 very poorly, although it phosphorylated MBP as well as full-length SAPK3 (1–367) (Figure 5A). Secondly, preincubation of SAP90 with

Figure 4 Phosphorylation *in vitro* of SAP90 at Thr²⁸⁷ and Ser²⁹⁰ by SAPK3/p38 γ and ERK2

GST-SAP90 was phosphorylated by 2.0 units/ml of SAPK3/p38 γ (A) or ERK2 (B) to 2.0 mol of phosphate/mol of substrate, then digested with chymotrypsin and chromatographed on a Vydac 218TP54 C₁₈ column as indicated in the Materials and methods section. Three peaks of ³²P radioactivity were obtained: one minor peak termed P0 and two major peaks termed P1 and P2. The acetonitrile gradient is shown by the fine line. All three peaks were examined by phospho-amino acid analysis: inset lanes (PS, phospho-serine; PT, phospho-threonine; PY, phospho-tyrosine).

synthetic peptides corresponding to the six or eight C-terminal amino acids of rat SAPK3/p38 γ prevented SAP90 phosphorylation (but not MBP) by SAPK3/p38 γ , but not by SAPK4/p38 δ (Figure 5B). Moreover, preincubation of wild-type rat SAPK3/p38 γ with an antibody raised against the 16 C-terminal amino acids, prevented phosphorylation of SAP90, but not MBP (results not shown).

In contrast with phosphorylation by SAPK3/p38 γ , phosphorylation of SAP90 by ERK2 or SAPK4/p38 δ was not diminished by preincubation of SAP90 with a peptide corresponding to the ten C-terminal residues of SAPK3/p38 γ (which suppressed phosphorylation of SAP90 by SAPK3/p38 γ by > 90%; results not shown).

Generation of phospho-specific antibodies that recognize SAP90 phosphorylated at Thr²⁸⁷ and Ser²⁹⁰

We generated two different phospho-specific antibodies that recognize SAP90 phosphorylated at sites Thr²⁸⁷ or Ser²⁹⁰. Their specificity was established by the finding that they only recognize GST-SAP90 after being phosphorylated *in vitro* by SAPK3/p38 γ and did not recognize the non-phosphorylated form of SAP90 (Figure 6). Furthermore, the recognition of phosphorylated SAP90 was abolished when the antibody was incubated with the phosphopeptide used to raise it, but not the non-phosphorylated form of this peptide or the phosphopeptide corresponding to the other phosphorylation site (Figure 6). However, the antibody Phos-Ser²⁹⁰ was specific at recognizing phosphorylated Ser²⁹⁰ in phospho-SAP90 only in the presence of the Ser²⁹⁰ peptide (Figure 6); for this reason we will use this antibody always in the presence of the unphosphorylated Ser²⁹⁰ peptide.

Cellular stresses induce phosphorylation of SAP90

To examine whether SAP90 becomes phosphorylated at Thr²⁸⁷ and Ser²⁹⁰, HEK-293 cells were transfected with GST-tagged SAP90 and exposed to cellular stresses that trigger the activation of SAPK3/p38 γ and SAPK2a/p38 α . We exposed cells to osmotic shock (0.5 M sorbitol) or UV-C (200 J/m²; Figure 7A). Activation of SAPK3/p38 γ by sorbitol was observed after 5 min, whereas activation of SAPK2a/p38 α was also observed after 5 min, but reached its maximum only at 60 min. Activation of SAPKs by UV-C was observed at 15 min, was maximal at 30 min and could still be detected at 60 min. Phosphorylation of SAP90 at Ser²⁹⁰ followed similar kinetics to activation of SAPK3/p38 γ (Figure 7A).

We found that Thr²⁸⁷ is phosphorylated in HEK-293 cells (Figure 7B) and PC12 cells (results not shown), even in the absence of stimulation. Treatment of SAP90 from these cells with the protein serine/threonine phosphatase 2A completely abolishes recognition by the anti-phospho-Thr²⁸⁷ antibody, confirming that Thr²⁸⁷ is phosphorylated in cells (Figure 7B). Moreover, the Phos-Thr²⁸⁷ antibody only recognizes SAP90 which is phosphorylated at Thr²⁸⁷ and does not recognize the unphosphorylated form

Figure 5 Regulation of SAP90 phosphorylation by the four C-terminal amino acids of SAPK3/p38 δ

(A) GST-SAP90 or MBP, both at 1 μ M, were phosphorylated for the different time periods indicated with 2.0 units/ml of either GST-SAPK3(1-367) or GST-SAPK3(1-363). Results are shown as means $\pm$ S.E.M. for four experiments. (B) GST-SAP90 (black and grey bars) or MBP (white bars), each at 1 μ M, were incubated for 30 min at room temperature with synthetic peptides (300 μ M), corresponding to the C-terminal six (PKETAL) or eight (RVPKETAL) amino acids of rat SAPK3. Either 0.2 units/ml GST-SAPK3 (black bars) or 0.2 units/ml GST-SAPK4 (grey bars) was added and the reactions were initiated with Mg[γ -³²P]ATP. Substrate phosphorylation is plotted as a percentage of that measured in the absence of each peptide. The concentration of each peptide required to inhibit GST-SAP90 phosphorylation by 50% was 50 μ M. Results are shown as means $\pm$ S.E.M. for triplicate determinations from a single experiment.

Figure 6 Characterization of antibodies that recognize phosphorylated and unphosphorylated SAP90

GST-SAP90 was phosphorylated by 2 units/ml of SAPK3/p38 γ . Different amounts of unphosphorylated (SAP90) and phosphorylated (pSAP90) GST-SAP90 were spotted on to a nitrocellulose membrane. The membranes were incubated with 1 μ g/ml of the SAP90 antibody that recognizes both the unphosphorylated and phosphorylated protein or an antibody called Phos-Ser²⁹⁰, raised against the peptide corresponding to residues 285–295 of SAP90 phosphorylated at Ser²⁹⁰, or an antibody called Phos-Thr²⁸⁷, raised against the peptide corresponding to residues 282–292 of SAP90 phosphorylated at Thr²⁸⁷. Immunoblots were prepared in the absence of any competing peptide (none) or in the presence of the phosphopeptide immunogen (Phos-Ser²⁹⁰ peptide for Phos-Ser²⁹⁰ antibody or Phos-Thr²⁸⁷ peptide for Phos-Thr²⁸⁷ antibody) or in the presence of the unphosphorylated peptide immunogen (Ser²⁹⁰ peptide for Phos-Ser²⁹⁰ antibody or Thr²⁸⁷ peptide for Phos-Thr²⁸⁷ antibody) or in the presence of phosphopeptide immunogen for the other site (Phos-Thr²⁸⁷ peptide for Phos-Ser²⁹⁰ antibody or Phos-Ser²⁹⁰ peptide for Phos-Thr²⁸⁷ antibody). Peptides were at 100 μ g/ml.

(Figure 6) or a SAP90 mutation in which Thr²⁸⁷ has been mutated to alanine (Figure 7B).

We have also examined the phosphorylation of endogenous SAP90 immunoprecipitated from PC12 cells after exposure to UV-C or sorbitol (Figure 8B). SAPKs are activated and SAP90 becomes phosphorylated at Ser²⁹⁰ under these conditions (Figure 8B).

To get information about which kinase(s) is/are responsible for SAP90 phosphorylation, we incubated cells with SB 203580 and/or PD 184352, before exposure to UV-C or osmotic shock. SB 203580 is a relatively specific inhibitor of SAPK2a/p38 α and SAPK2b/p38 β , whereas PD 184352 is a potent inhibitor of the ERK pathway. Neither compound had any significant effect on the phosphorylation of endogenous SAP90 by these cellular stresses (Figure 8). In contrast, SB 203580 prevented the activation of MAPKAP-K2, a substrate for SAPK2a/p38 α and SAPK2b/p38 β , as judged by suppression of the phosphorylation of Hsp27, one of its substrates [32] (Figure 8A), and PD 184352 prevented the activation of ERK1/ERK2 (Figure 8B). These results suggest that phosphorylation of SAP90 at Ser²⁹⁰ could be mediated by SAPK3/p38 γ , since UV-C and sorbitol treatment did not activate SAPK4/p38 δ or ERK2 in these cells (results not shown), which are the other two enzymes that phosphorylate SAP90 *in vitro*.

Mitogens induce phosphorylation of SAP90 in cells

SAP90 is also an *in vitro* substrate for ERK2 (Table 1 and Figure 4B). Therefore, we investigated whether stimuli that activate this kinase *in vivo*, such as EGF or PMA, could phosphorylate SAP90. Interestingly, we found that SAP90 is phosphorylated at Ser²⁹⁰ after EGF or PMA treatment of HEK-293 cells (Figure 9A) and PC12 cells (Figure 9B).

As expected, the activation of ERK1/ERK2 was inhibited by incubating cells with PD 184352 before stimulation with EGF or PMA (Figure 9). After subjecting cells to mitogens, the phosphorylation of SAP90 is probably mediated by ERK1/ERK2 and not by SAPKs, as PD 184352, but not SB 203580, prevented the phosphorylation of SAP90 under these conditions (Figure 9).

Stress-induced phosphorylation of SAP90 is blocked *in vivo* by a peptide corresponding to the C-terminal residues of SAPK3/p38 γ

Since preincubation of SAP90 with peptides corresponding to the C-terminal amino acids of SAPK3/p38 γ prevented *in vitro*

Figure 7 Phosphorylation of SAP90 at Ser²⁹⁰ after activation of SAPK3/p38 γ in HEK-293 cells by cellular stress

(A) After transfection of HEK-293 cells with a plasmid encoding GST-SAP90, cells were exposed to UV-C (200 J/m²) or to osmotic shock (0.5 M sorbitol), after the time period indicated; 10 μ g of cell lysates were denatured, electrophoresed on 7% gel and immunoblotted using an antibody that recognizes SAP90 phosphorylated at Ser²⁹⁰ (Phos-Ser²⁹⁰), in the presence of 100 μ g/ml Ser²⁹⁰ peptide, and an antibody that recognizes unphosphorylated and phosphorylated SAP90 antibody. To examine SAPK activation, 50 μ g of cell lysates were used in the immunoblot. SAPK2a/p38 α phospho-specific antibody also recognizes phosphorylated SAPK3/p38 γ (see the Materials and methods section). (B) GST-SAP90 from 50 μ g of HEK-293 lysates was bound to GSH-Sepharose beads. Pellets were treated with or without 60 m-units/ml of protein serine/threonine phosphatase 2A (PP2A) for 30 min at 30 °C. Samples were denatured, electrophoresed and immunoblotted using an antibody that recognizes SAP90 phosphorylated at Thr²⁸⁷ (Phos-Thr²⁸⁷) and an antibody that recognizes unphosphorylated and phosphorylated SAP90 antibody.

Figure 8 Lack of inhibition of SAP90 stress-induced phosphorylation by the SAPK2 α /p38 α or the ERK pathways inhibitors

Cells were incubated for 1 h without or with 10 μ M SB 203580 and/or 5 μ M PD 184352, then exposed for 15 min to 0.5 M sorbitol or to UV-C radiation (200 J/m²), followed by a 30 min incubation. (A) Aliquots of lysates from HEK-293 cells overexpressing SAP90 (50 μ g of protein) were subjected to SDS/PAGE and immunoblotted with an antibody that recognizes Hsp27 at Ser¹⁵. (B) PC12 cells were lysed and endogenous SAP90 was immunoprecipitated with SAP90 antibody from aliquots of the extracts (5 mg of protein), denatured, electrophoresed and immunoblotted as described in the legend to Figure 7.

phosphorylation of SAP90 by SAPK3/p38 γ (Figure 5B), we constructed a cell-permeant peptide by fusing the last nine residues of SAPK3/p38 γ to the cell-membrane transduction domain of the HIV-1 Tat protein [33,34]. This resulted in the 20-amino-acid peptide Tat-SAPK3C, which, we anticipated, would dissociate the SAP90-SAPK3/p38 γ complex in cells, thus reducing the phosphorylation of SAP90 by cellular stresses. First, we established that the Tat-SAPK3C peptide penetrated cells by showing that HEK-293 cells treated with dansyl-Tat-SAPK3C exhibited intracellular fluorescence indicating peptide uptake (Figures 10A and 10B). Dansyl-Tat-SAPK3C accumulation in HEK-293 cells was detected at 40 min of application and remained detectable after 4 h (results not shown). Secondly, we found that incubation of cells with Tat-SAPK3C peptide significantly decreased co-immunoprecipitation of SAP90 with SAPK3/p38 γ (Figure 10C). Finally, we examined whether the phosphorylation of SAP90 in HEK-293 cells is affected by incubation of the cells with Tat-SAPK3C before exposure to stress or mitogen. Preincubation with the peptide Tat-SAPK3C did not prevent the activation of SAPK3/p38 γ nor SAPK2 α /p38 α by cellular stresses, such as UV-C or osmotic shock, although it significantly decreased the phosphorylation of SAP90 (Figure 10D). In contrast, phosphorylation of the SAPK2 α /p38 α substrate MAPKAP-K2 was unaffected by Tat-SAPK3C (Figure 10D), or the activation of JNK or the phosphorylation of its substrate c-Jun (results not shown), indicating

Figure 9 Inhibition of mitogen-induced phosphorylation of SAP90 by the ERK pathway inhibitor

Cells were incubated for 1 h without or with 10 μ M SB 203580 and/or 5 μ M PD 184352 and then exposed for 15 min to 100 ng/ml EGF or 400 ng/ml PMA (TPA) for 15 min (PC12 cells) or 30 min (HEK-293 cells). (A) Aliquots of lysates from HEK-293 cells overexpressing SAP90 (10 μ g of protein) were subjected to SDS/PAGE and immunoblotted with the antibodies described above. (B) PC12 cells were lysed and endogenous SAP90 was immunoprecipitated with a SAP90 antibody from aliquots of the extracts (5 mg of protein), denatured, electrophoresed and immunoblotted, as described in the legend to Figure 7.

that SAPK3/p38 γ is responsible for the phosphorylation of SAP90 at Ser²⁹⁰ under these conditions. On the other hand, phosphorylation of SAP90 and activation of ERK1/ERK2 by EGF were not affected by preincubation of the cells with Tat-SAPK3C (Figure 10D).

DISCUSSION

The present study shows that SAP90 (also called PSD-95) is a good *in vitro* substrate for the MAPK family members ERK2

Figure 10 Inhibition of the *in vivo* stress-induced phosphorylation of SAP90 by a peptide corresponding to the C-terminal residues of SAPK3/p38 γ

(A) Visualization of intracellular accumulation of Dansyl-TatSAPK3C peptide 90 min after application to HEK-293 culture; (B) phase contrast of the same region. (C) Co-immunoprecipitation of SAP90 and SAPK3/p38 γ from HEK-293 cells incubated with or without the TatSAPK3 peptide as described in the Materials and methods section. (D) HEK-293 cells expressing GST-SAP90 were incubated for 1 h with or without 400 μ M TatSAPK3C peptide, then exposed for 15 min to 0.5 M sorbitol, 100 ng/ml EGF or UV-C radiation (200 J/m²), followed by a 30 min incubation. SAP90 was precipitated with GSH-Sepharose from aliquots of the extracts (20 μ g of protein), denatured, electrophoresed and immunoblotted as described in the legend to Figure 7. To examine SAPK activation, 50 μ g of cell lysates were immunoblotted with an antibody that recognizes MAPKAP-K2 phosphorylated at Thr³³⁴ or with the SAPK2a/p38 α phospho-specific antibody recognizing phosphorylated SAPK3/p38 γ (see the Materials and methods section).

and SAPK3/p38 γ , which phosphorylate SAP90 at Thr²⁸⁷ and Ser²⁹⁰. Furthermore, we also show that SAP90 is phosphorylated *in vivo* at Thr²⁸⁷ and that Ser²⁹⁰ becomes phosphorylated following exposure to cellular stresses or mitogens. These findings establish that SAP90 is a physiological substrate for one or more proline-directed kinases. Phosphorylation of SAP90 at Ser²⁹⁰ after EGF

or PMA treatment was largely blocked by pretreatment with the ERK pathway inhibitor PD 184352, suggesting that ERK1/ERK2 is probably responsible for phosphorylation at this site. Moreover, SAP90 also became phosphorylated at Ser²⁹⁰ in response to osmotic shock or UV-C radiation. The time course of Ser²⁹⁰ phosphorylation paralleled the activation of SAPK3/p38 γ and was not abolished by incubation of the cells before stimulation with PD 184352 and/or SB 203580. These results indicate that ERK1, ERK2, SAPK2a/p38 α and SAPK2b/p38 β are not rate-limiting for the phosphorylation of SAP90 under these conditions. Activation of ERK5, also triggered by osmotic shock [35,36], is inhibited by PD 184352 at high concentrations [36]. However, ERK5 does not seem to be involved in the phosphorylation of SAP90 at Ser²⁹⁰, since it was activated very poorly in the cells used in this study (results not shown) and the phosphorylation of SAP90 was not inhibited by high (10 μ M) doses of PD 184352 which inhibit the activation of ERK5. It is unlikely that other SAPKs resistant to SB 203580 and/or PD 184352 are relevant SAP90 kinases. Thus SAPK4/p38 δ was not activated in the cells during the present study (results not shown) and SAP90 was only poorly phosphorylated by JNKs *in vitro*. In contrast, SAPK3/p38 γ is a strong candidate kinase for phosphorylating SAP90 at Ser²⁹⁰ under stressful conditions, given that its activation was similar to Ser²⁹⁰ phosphorylation and its activity is not inhibited by SB 203580 and PD 184352. Moreover, we also show that the phosphorylation of SAP90 by SAPK3/p38 γ , but not by ERK2 or SAPK4/p38 δ , is blocked by preincubation of SAP90 with synthetic peptides corresponding to the C-terminal six or eight residues of SAPK3/p38 γ . This blocking peptide can be transduced into the cells by fusing it to a cell-permeant peptide from the Tat protein. We show that preincubation of cells with the TatSAPK3C peptide before subjecting the cells to osmotic shock or UV-C radiation, but not to EGF, prevented the phosphorylation of SAP90, but did not block SAPK2a/p38 α or ERK1/ERK2 activity. These results strongly suggest that SAPK3/p38 γ is the stress-activated kinase that phosphorylates SAP90 at Ser²⁹⁰.

In a previous study, we reported that SAPK3/p38 γ is the only MAPK family member that is capable of binding to the PDZ domain of α 1-syntrophin through its C-terminal sequence (-ETXL) [11]. In the present study, we show that SAPK3/p38 γ associates with SAP90 in brain extracts and in cultured cells. Furthermore, SAP90 and SAPK3/p38 γ were found to exhibit an overlapping distribution in cerebellar granule cells and differentiated PC12 cells and to co-localize at synaptic sites between hippocampal neurons. *In vitro*, SAPK3/p38 γ binds preferentially to the PDZ domain 3 of SAP90. The C-terminal residue of SAPK3/p38 γ is a leucine (at position 0), although crystallographic studies have shown that valine (at position 0) is preferred by PDZ3 of SAP90, which can also bind to sequences ending with isoleucine or leucine [15,16]. Intriguingly, we also show that the phosphorylation of SAP90 by SAPK3/p38 γ depends on binding to the PDZ domain of SAP90. The C-terminal sequence of SAPK3/p38 γ thus provides a mechanism both for its selective targeting to subcellular sites and for determining its substrate specificity.

The present study raises the question of the physiological role of the SAPK3/p38 γ -SAP90 association. There are several other examples of protein kinases that bind to PDZ domains. Thus p70 S6 kinase has been shown to associate through its C-terminal sequence with the PDZ domain of neurabin, suggesting that the neuronal-specific neurabin may target p70 S6 kinase to nerve terminals [37]. The eye-PKC (protein kinase C) can bind to PDZ domains and the C-terminus of PKC is critical for interaction with both the PDZ2 and PDZ4 domains of INAD (inactivation-no after potential D), which is a multidomain scaffolding protein comprising five PDZ domains that are required for co-ordinating

the molecular activities of phototransduction [38–41]. Moreover, the C-terminal sequence of PKC α interacts with the PDZ domain of the PICK (proteins that interact with protein C-kinase) [42]. PICK1 interacts with the kainate receptor subunits GluR5(2b), GluR5(2c) and GluR6, and targets PKC α to phosphorylate kainate receptors, with this phosphorylation being required for maintaining synaptic responses mediated by kainate receptors [43].

Our findings also raise the question of the role played by phosphorylation in regulating the localization of SAP90 and its binding to other proteins. To our knowledge, this is the first example of a PDZ domain containing protein being shown to be selectively phosphorylated *in vivo*. Although the functional importance of Ser²⁹⁰ phosphorylation in SAP90 is not known, there are several examples wherein the phosphorylation of residues near the C-terminal tails of proteins regulates their PDZ interaction. For instance, the –2 serine of the inward rectifier potassium channel Kir2.3 is phosphorylated by protein kinase A and this phosphorylation abolishes the interaction of Kir2.3 with the PDZ domains of SAP90 [15]. Furthermore, phosphorylation of the –2 serine of the β 2-adrenergic receptor by G-protein-coupled receptor kinase 5 disrupts the binding of the receptor to the PDZ domain of NHERF (Na⁺/H⁺-exchanger regulatory factor)/EBP50 [(ERM (ezrin, radixin, moesin)-binding phosphoprotein 50) [44,45]. Moreover, the –3 serine of the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunit GluR2 can be phosphorylated by PKC, and this modification prevents GluR2 from binding to the glutamate receptor interacting protein, but not to PICK [46,47]. One of the first described interacting partners for SAP90 was the shaker voltage-gated potassium channel (Kv1.4) [48]. This channel was found to bind the first two PDZ domains in SAP90. Interestingly, when co-expressed in heterologous cells, SAP90 promoted the formation of cell-surface clusters of Kv1.4 via a head-head N-terminal multimerization mechanism [49]. Given that SAPK3/p38 γ phosphorylates residues in the N-terminal half of SAP90, we evaluated whether the activation of SAPK3/p38 γ affected the binding and clustering of Kv1.4 by SAP90. However, even under cellular stress (0.5 M sorbitol), no effect was seen (results not shown). It thus remains to be shown how the activation of synaptic SAPK3/p38 γ functionally affects SAP90 and its binding partners.

Results of the present study demonstrate that SAP90 is a novel binding partner for SAPK3/p38 γ and a novel *in vivo* substrate for SAPK3/p38 γ and ERK1/ERK2. They suggest that phosphorylation of SAP90, and/or binding to SAPK3/p38 γ , may play a role in regulating protein–protein interactions at the synapse, in response to stresses or mitogen-related signalling events.

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