Stress- and mitogen-induced phosphorylation of the synapse-associated protein SAP90/PSD-95 by activation of SAPK3/p38γ and ERK1/ERK2

Guadalupe SABIO*†, Suzana REUVER‡, Carmen FEIJOO*, Masato HASEGAWA§[1], Gareth M. THOMAS*[2], Francisco CENTENO†, Sven KUHLENDAHL‡, Sergio LEAL-ORTIZ‖, Michel GOEDERT§, Craig GARNER‡‖ and Ana CUENDA*[3]

*MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, U.K., †Departamento Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Extremadura, 10071 Cáceres, Spain, ‡Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL 39294, U.S.A., §MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K., and ‖Nancy Pritzker Laboratory, 1201 Welch Road, Room P106, Stanford, CA 94305-5485, U.S.A.

SAPK3 (stress-activated protein kinase-3, also known as p38γ) 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 α1-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/p38γ and the ERK (extracellular-signal-regulated protein kinase). SAPK3/p38γ binds preferentially to the third PDZ domain of SAP90 and phosphorylates residues Thr^{287} and Ser^{290} *in vitro*, and Ser^{290} in cells in response to cellular stresses. Phosphorylation of SAP90 is dependent on the binding of SAPK3/p38γ to the PDZ domain of SAP90. It is not blocked by SB 203580, which inhibits SAPK2a/p38α and SAPK2b/p38β but not SAPK3/p38γ, 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/p38γ with SAP90. ERK2 also phosphorylates SAP90 at Thr^{287} and Ser^{290} *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/p38γ co-localize and they are co-immunoprecipitated from brain synaptic junctional preparations. These results demonstrate that SAP90 is a novel binding partner for SAPK3/p38γ, a first physiological substrate described for SAPK3/p38γ 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 mitogenrelated stimuli.

Key words: extracellular-signal-regulated kinase (ERK), phosphorylation, stress, stress-activated protein kinase-3 (SAPK3)/p38γ, 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/p38α, SAPK2b/p38β, SAPK3/p38γ [also known as ERK6 (extracellular-signal-regulated kinase 6)] and SAPK4/p38δ. Identification of physiological substrates for SAPK2a/p38α and SAPK2b/p38β 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/p38α and SAPK2b/p38β include other protein kinases, as well as several transcription factors [3]. SAPK3/p38γ and SAPK4/p38δ 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/p38δ [6–8], whereas the activating transcription factor-2, microtubule-associated protein Tau and the protein α1-syntrophin are substrates of SAPK3/p38γ *in vitro* [9–12].

Phosphorylation of α1-syntrophin by SAPK3/p38γ depends on the interaction of the C-terminal sequence-ETXL of the kinase with the PDZ domain of α1-syntrophin. In skeletal muscles, SAPK3/p38γ and α1-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/p38γ binds through its C-terminal sequence to the PDZ domain of α1-syntrophin and that the phosphorylation of α1-syntrophin by SAPK3/p38γ 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.

[1] Present address: Department of Molecular Neurobiology, Tokyo Institute of Psychiatry, Tokyo 156-8585, Japan.

[2] Present address: Department of Neuroscience, School of Medicine, Johns Hopkins University, Howard Hughes Medical Institute, 725 N. Wolfe Street, PCTB 904, Baltimore, MD 21205, U.S.A.

[3] To whom correspondence should be addressed (e-mail a.i.cuenda@dundee.ac.uk).

© 2004 Biochemical Society

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

Although SAPK3/p38γ 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/p38γ will be found to interact with PDZ domains of proteins other than α1-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-CO_2H, similar to the C-terminus of SAPK3/p38γ [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 neuroligin, 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/p38γ 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/p38γ and SAP90 co-localize at synapses and interact in synaptic junctional preparations. These findings indicate that SAPK3/p38γ-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 AMTPTphosSPRRYS (residues 285–295) of SAP90 were coupled with BSA and keyhole limpet haemocyanin and injected into sheep at Diagnostics 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 μg/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/p38γ in immunofluorescence staining was generated by injecting sheep with peptides corresponding to the N- and C-terminal regions of SAPK3/p38γ. The antibody was affinity-purified on a GST–SAPK3 antigen-Sepharose column.

An antibody that recognizes the Hsp27 (heat-shock protein 27; phosphorylated at Ser^{15}) was described previously [7]. Antibodies that recognize SAPK2a/p38α phosphorylated at Thr^{180} and Tyr^{182} (these antibodies also recognize phosphorylated SAPK2b/p38β, SAPK3/p38γ and SAPK4/p38δ), ERK1/ERK2, phospho-ERK1/ERK2 (Thr^{202}/Tyr^{204}) and phospho-MAPKAP-K2 (where MAPKAP stands for MAP kinase-activated protein; Thr^{334}) were purchased from New England Biolabs (Hitchin, Herts., U.K.) and a SAPK2a/p38α 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(ΔC)(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),

© 2004 Biochemical Society

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 μg/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/$NiCl_2$. 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 μg/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 % O_2/5 % CO_2, 1 μg DNA (previously incubated for 15 min at room temperature with 6 μl of FuGENE 6 reagent in 100 μl 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/m^2), 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 μM SB 203580 and/or 5 μM PD 184352, or with 400 μM (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 μg 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 × 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/p38γ and SAP90 were immunostained by using 12 μg/ml SAPK3 antibody or 4 μg/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 × 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/p38γ and other MAPK family members

SAPK3/p38γ and other MAPK family members were assayed routinely using MBP as substrate [9]. Phosphorylation of SAP90

© 2004 Biochemical Society

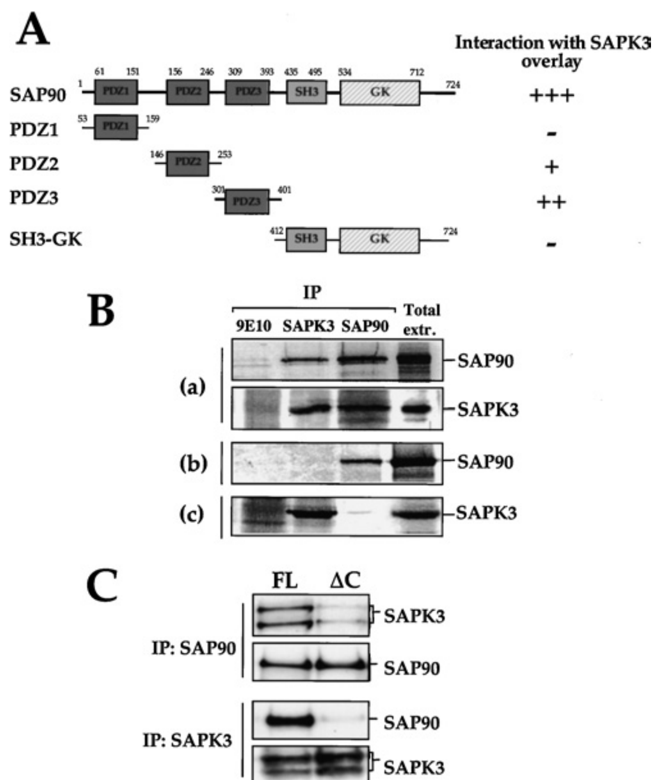

Figure 1 Interaction of SAPK3/p38γ with SAP90

(**A**) Interaction of SAPK3/p38γ with SAP90/PSD-95. An overlay binding assay was used to assess the interaction between SAPK3/p38γ 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/p38γ. (**B**) Co-immunoprecipitation of SAP90 and SAPK3/p38γ 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/p38γ with SAP90. HEK-293 cells were co-transfected with GST-SAP90 and GFP-SAPK3 (FL, full length: 1–367) or GFP-SAPK3 (ΔC, lacking the last four amino acids: 1–363). GST-SAP90 from the lysates was bound to GSH–Sepharose 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 μM) was incubated for 1 h at 30 °C with activated GST-SAPK3 (2.0 units/ml), 10 mM magnesium acetate and 100 μM [γ-^{32}P]ATP in a total volume of 200 μl 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 C_{18} column equilibrated with 10 mM ammonium acetate (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.

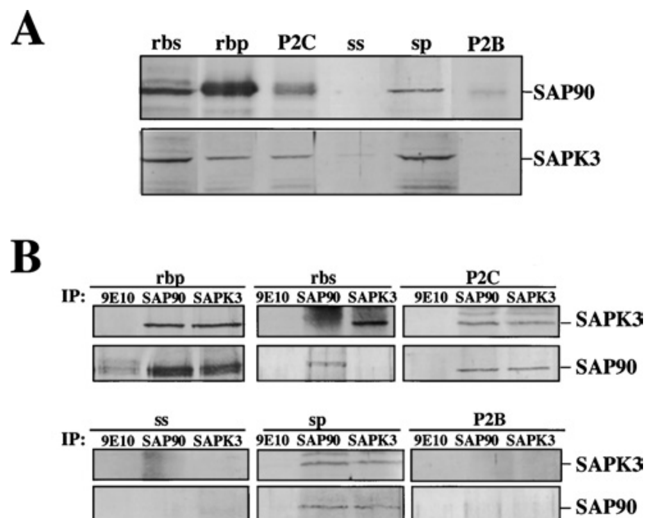

Figure 2 Expression and interaction of SAP90 and SAPK3 in subcellular fractions of adult rat brain

(**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/p38γ and mouse antibody against SAP90. SAP90 and SAPK3 co-immunoprecipitate in synaptic plasma membrane fractions, but not in endoplasmic reticulum, Golgi or soluble fractions.

RESULTS

SAPK3/p38γ interacts with SAP90

We have shown previously that the C-terminus of SAPK3/p38γ binds to the PDZ domain of the protein α1-syntrophin [11]. Since SAP90 is the prototype of a PDZ domain-containing protein, we investigated whether SAPK3/p38γ interacts with SAP90 *in vitro*. An overlay filter-binding assay was used to examine the domains of SAP90 that interact with SAPK3/p38γ (Figure 1A). Full-length SAP90 bound strongly to SAPK3/p38γ, 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/p38γ. These results suggest that as with α1-syntrophin [11], SAP90 binds SAPK3/p38γ through one of its PDZ domains.

To assess whether SAPK3/p38γ could bind SAP90 as a full-length protein in a cellular environment, we transfected HEK-293 cells with SAPK3/p38γ and SAP90 either alone or together. Cell lysates were subjected to immunoprecipitation with antibodies against SAPK3/p38γ, SAP90 or antibodies against a c-myc epitope (9E10) and Western-blotted (Figure 1B). In these experiments, antibodies to SAPK3/p38γ were not only able to immunoprecipitate SAPK3/p38γ, but, when present, also SAP90. Similarly, antibodies against SAP90 precipitated SAPK3/p38γ as

© 2004 Biochemical Society

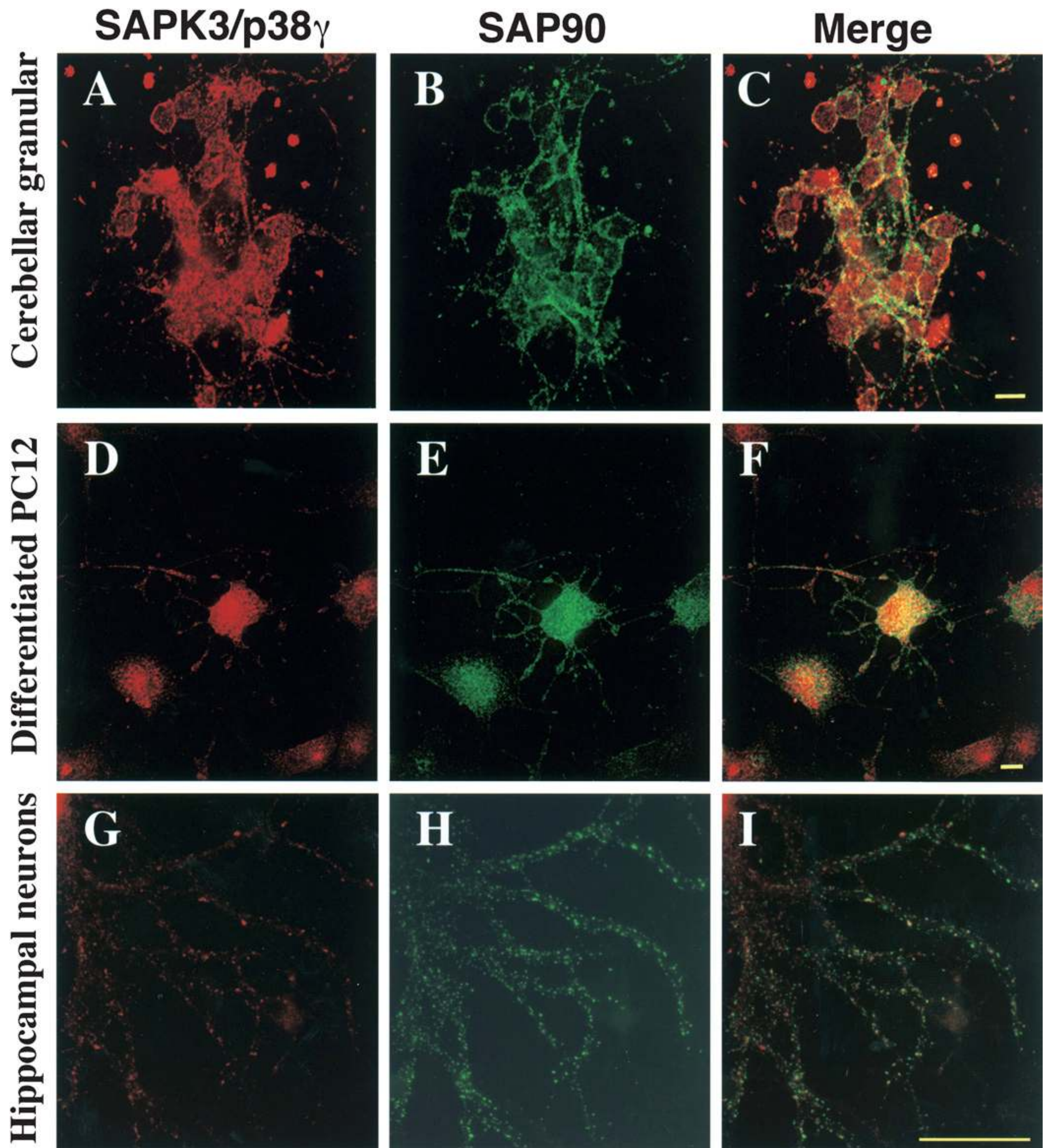

Figure 3 Distribution of SAPK3/p38γ and SAP90 in cells

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/p38γ 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 μm.

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/p38γ 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 (ΔC, 1–363: lacking the last four amino acids). As predicted, SAP90 only co-immunoprecipitated full-length SAPK3/p38γ, but not

© 2004 Biochemical Society

SAPK3/p38γ with the C-terminal truncation SAPK3(ΔC) (Figure 1C). These results indicate that the last four amino acids of SAPK3/p38γ, 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/p38γ suggested that this interaction might be relevant *in vivo*. To test this hypothesis, we initially examined whether SAPK3/p38γ 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/p38γ. 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/p38γ interact within these membrane compartments, we performed immunoprecipitations of each fraction with antibodies against SAPK3/p38γ or SAP90 (5′2d). SAPK3/p38γ 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/p38γ may interact with each other while associated with post-synaptic plasma membranes and not necessarily while in the cytosol.

SAP90 co-localizes with SAPK3/p38γ in neurons

The ability of SAP90 and SAPK3/p38γ 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/p38γ 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/p38γ immunoreactivity was found in the nucleus, cytoplasm and along neuritic processes. Similarly, in cerebellar granule cells, SAPK3/p38γ 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/p38γ 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/p38γ 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/p38γ, whereas the former is consistent with the presence of SAPK3/p38γ in the cytosol. It should be noted that, whereas SAPK3/p38γ 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/p38γ staining, the patterns observed were abolished by incubation of the SAPK3 antiserum with recombinant SAPK3/p38γ or with the N- and C-terminal peptides (results not shown). Furthermore, the SAPK3/p38γ localization was confirmed with three different antibodies (results not shown).

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 μM SAP90 (%) relative to that of 1 μM MBP (= 100 %)
SAPK3/p38γ	100 ± 10
SAPK4/p38δ	63 ± 4
MAPK2/ERK2	48 ± 7
SAPK2a/p38α	2 ± 1
SAPK2b/p38β	5 ± 1

(b)

[Kinase] (0.1 unit/ml)	Rate of phosphorylation of 1 μM SAP90 (%) relative to that of 1 μM ATF2 (= 100 %)
SAPK3/p38γ	100 ± 5
JNK2α	6.2 ± 1
JNK3	4.0 ± 0.5

SAP90 is phosphorylated *in vitro* by SAPK3/p38γ 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/p38γ (Table 1). ERK2 and SAPK4/p38δ phosphorylated SAP90 at lower rates, whereas SAPK2a/p38α, SAPK2b/p38β and JNK phosphorylated it weakly (Table 1).

At higher concentrations, SAPK3/p38γ (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/p38γ was then digested with chymotrypsin and the resulting peptides were chromatographed on a C_{18} column. Two major peaks of ^{32}P 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 diphosphorylated at Thr^{287} and Ser^{290}. Residues Thr^{287} and Ser^{290} are located between PDZ domains 2 and 3 of SAP90.

The sites phosphorylated in SAP90 by ERK2 (Figure 4) and SAPK4/p38δ (results not shown) were also analysed. P1 and P2 were the major peaks and both contained residues Thr^{287} and Ser^{290}. P0 contained a peptide corresponding to residues 13–34, which was mono-phosphorylated at Ser^{25} (Figure 4).

Interestingly, the phosphorylation of SAP90 by SAPK3/p38γ *in vitro* was dependent on the extreme C-terminus of SAPK3/p38γ. This was demonstrated by the following observations. First, SAPK3(1–363), a form of SAPK3/p38γ 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

© 2004 Biochemical Society

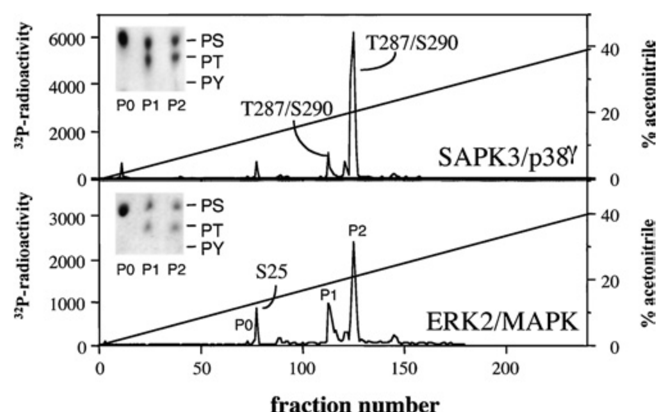

Figure 4 Phosphorylation *in vitro* of SAP90 at Thr^{287} and Ser^{290} by SAPK3/p38γ and ERK2

GST-SAP90 was phosphorylated by 2.0 units/ml of SAPK3/p38γ (**A**) or ERK2 (**B**) to 2.0 mol of phosphate/mol of substrate, then digested with chymotrypsin and chromatographed on a Vydac 218TP54 C_{18} column as indicated in the Materials and methods section. Three peaks of ^{32}P 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/p38γ prevented SAP90 phosphorylation (but not MBP) by SAPK3/p38γ, but not by SAPK4/p38δ (Figure 5B). Moreover, preincubation of wild-type rat SAPK3/p38γ 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/p38γ, phosphorylation of SAP90 by ERK2 or SAPK4/p38δ was not diminished by preincubation of SAP90 with a peptide corresponding to the ten C-terminal residues of SAPK3/p38γ (which suppressed phosphorylation of SAP90 by SAPK3/p38γ by $>90\%$; results not shown).

Generation of phospho-specific antibodies that recognize SAP90 phosphorylated at Thr^{287} and Ser^{290}

We generated two different phospho-specific antibodies that recognize SAP90 phosphorylated at sites Thr^{287} or Ser^{290}. Their specificity was established by the finding that they only recognize GST-SAP90 after being phosphorylated *in vitro* by SAPK3/p38γ 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-Ser^{290} was specific at recognizing phosphorylated Ser^{290} in phospho-SAP90 only in the presence of the Ser^{290} peptide (Figure 6); for this reason we will use this antibody always in the presence of the unphosphorylated Ser^{290} peptide.

Cellular stresses induce phosphorylation of SAP90

To examine whether SAP90 becomes phosphorylated at Thr^{287} and Ser^{290}, HEK-293 cells were transfected with GST-tagged SAP90 and exposed to cellular stresses that trigger the activation of SAPK3/p38γ and SAPK2a/p38α. We exposed cells to osmotic shock (0.5 M sorbitol) or UV-C (200 J/m^2; Figure 7A). Activation of SAPK3/p38γ by sorbitol was observed after 5 min, whereas activation of SAPK2a/p38α 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 Ser^{290} followed similar kinetics to activation of SAPK3/p38γ (Figure 7A).

We found that Thr^{287} 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-Thr^{287} antibody, confirming that Thr^{287} is phosphorylated in cells (Figure 7B). Moreover, the Phos-Thr^{287} antibody only recognizes SAP90 which is phosphorylated at Thr^{287} and does not recognize the unphosphorylated form

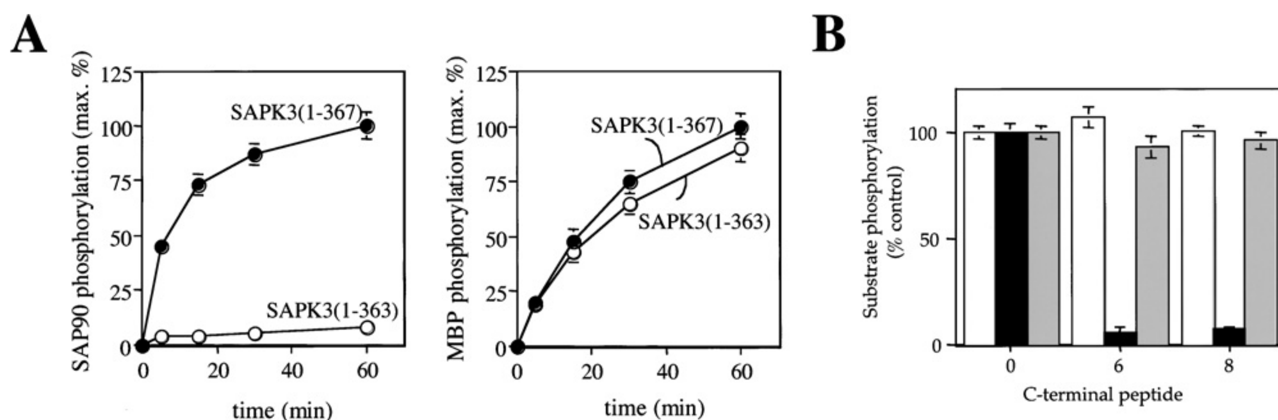

Figure 5 Regulation of SAP90 phosphorylation by the four C-terminal amino acids of SAPK3/p38δ

(**A**) GST-SAP90 or MBP, both at 1 μM, 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 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. 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-^{32}P]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 μM. Results are shown as means $\pm$ S.E.M. for triplicate determinations from a single experiment.

© 2004 Biochemical Society

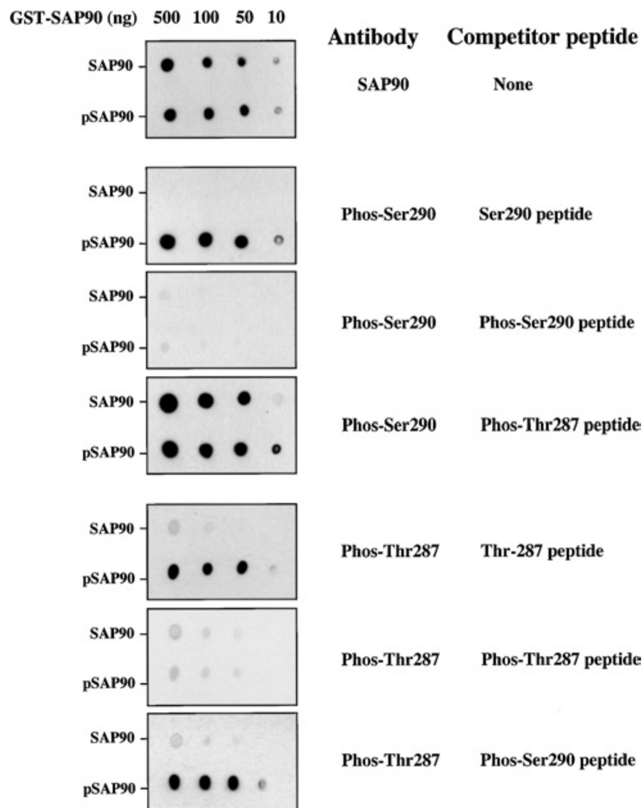

Figure 6 Characterization of antibodies that recognize phosphorylated and unphosphorylated SAP90

GST-SAP90 was phosphorylated by 2 units/ml of SAPK3/p38γ. Different amounts of unphosphorylated (SAP90) and phosphorylated (pSAP90) GST-SAP90 were spotted on to a nitrocellulose membrane. The membranes were incubated with 1 μg/ml of the SAP90 antibody that recognizes both the unphosphorylated and phosphorylated protein or an antibody called Phos-Ser^{290}, raised against the peptide corresponding to residues 285–295 of SAP90 phosphorylated at Ser^{290}, or an antibody called Phos-Thr^{287}, raised against the peptide corresponding to residues 282–292 of SAP90 phosphorylated at Thr^{287}. Immunoblots were prepared in the absence of any competing peptide (none) or in the presence of the phosphopeptide immunogen (Phos-Ser^{290} peptide for Phos-Ser^{290} antibody or Phos-Thr^{287} peptide for Phos-Thr^{287} antibody) or in the presence of the unphosphorylated peptide immunogen (Ser^{290} peptide for Phos-Ser^{290} antibody or Thr^{287} peptide for Phos-Thr^{287} antibody) or in the presence of phosphopeptide immunogen for the other site (Phos-Thr^{287} peptide for Phos-Ser^{290} antibody or Phos-Ser^{290} peptide for Phos-Thr^{287} antibody). Peptides were at 100 μg/ml.

(Figure 6) or a SAP90 mutation in which Thr^{287} 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 Ser^{290} 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/p38α and SAPK2b/p38β, 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/p38α and SAPK2b/p38β, 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 Ser^{290} could be mediated by SAPK3/p38γ, since UV-C and sorbitol treatment did not activate SAPK4/p38δ 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 Ser^{290} 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/p38γ

Since preincubation of SAP90 with peptides corresponding to the C-terminal amino acids of SAPK3/p38γ prevented *in vitro*

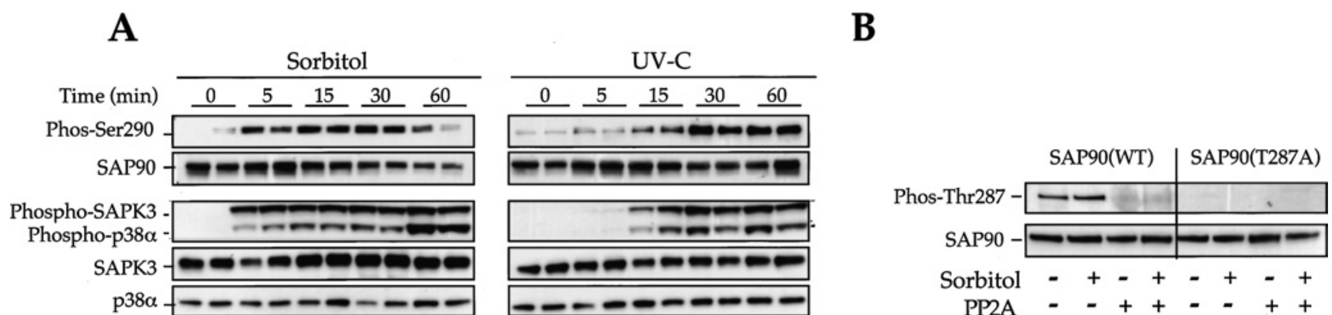

Figure 7 Phosphorylation of SAP90 at Ser^{290} after activation of SAPK3/p38γ in HEK-293 cells by cellular stress

(**A**) After transfection of HEK-293 cells with a plasmid encoding GST-SAP90, cells were exposed to UV-C (200 J/m^2) or to osmotic shock (0.5 M sorbitol), after the time period indicated; 10 μg of cell lysates were denatured, electrophoresed on 7 % gel and immunoblotted using an antibody that recognizes SAP90 phosphorylated at Ser^{290} (Phos-Ser^{290}), in the presence of 100 μg/ml Ser^{290} peptide, and an antibody that recognizes unphosphorylated and phosphorylated SAP90 antibody. To examine SAPK activation, 50 μg of cell lysates were used in the immunoblot. SAPK2a/p38α phospho-specific antibody also recognizes phosphorylated SAPK3/p38γ (see the Materials and methods section). (**B**) GST-SAP90 from 50 μg 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 Thr^{287} (Phos-Thr^{287}) and an antibody that recognizes unphosphorylated and phosphorylated SAP90 antibody.

© 2004 Biochemical Society

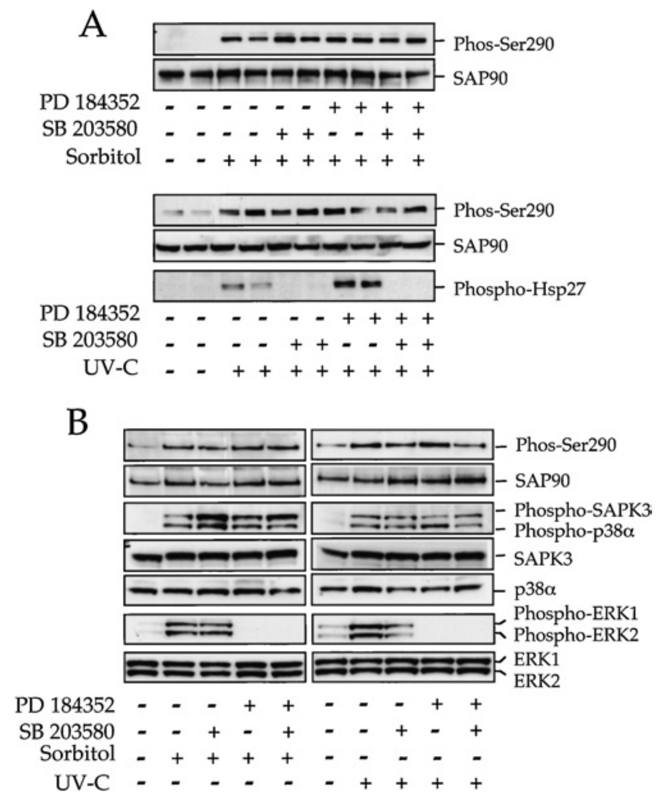

Figure 8 Lack of inhibition of SAP90 stress-induced phosphorylation by the SAPK2a/p38α or the ERK pathways inhibitors

Cells were incubated for 1 h without or with 10 μM SB 203580 and/or 5 μM PD 184352, 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. (**A**) Aliquots of lysates from HEK-293 cells overexpressing SAP90 (50 μg of protein) were subjected to SDS/PAGE and immunoblotted with an antibody that recognizes Hsp27 at Ser^{15}. (**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.

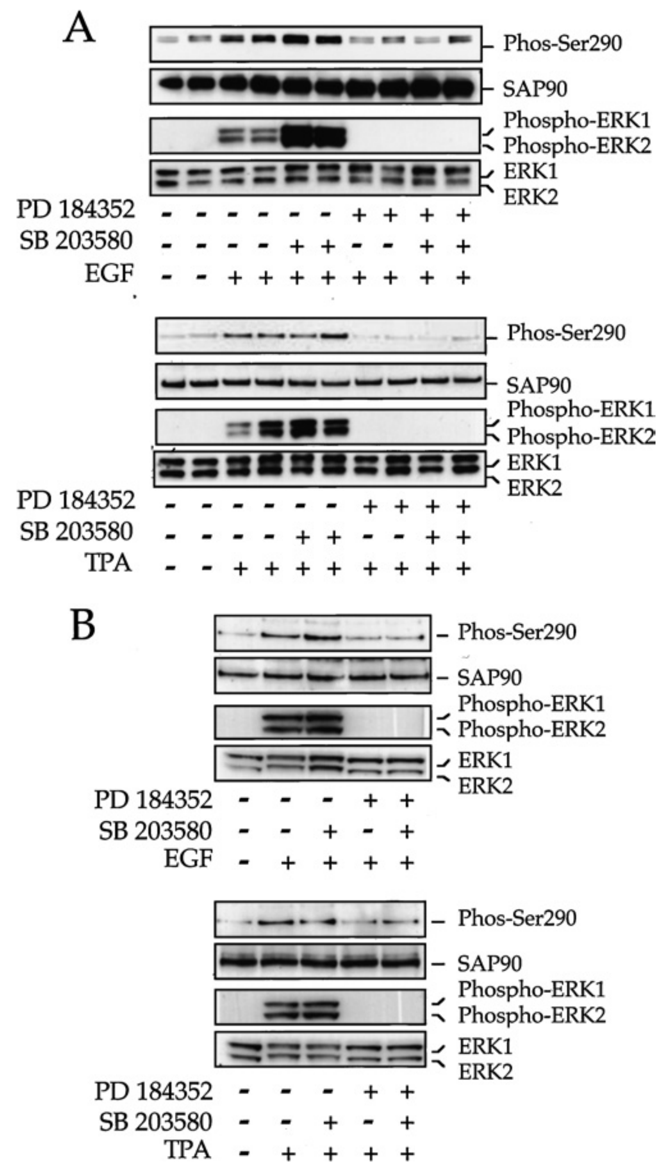

Figure 9 Inhibition of mitogen-induced phosphorylation of SAP90 by the ERK pathway inhibitor

Cells were incubated for 1 h without or with 10 μM SB 203580 and/or 5 μM 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 μg 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.

phosphorylation of SAP90 by SAPK3/p38γ (Figure 5B), we constructed a cell-permeant peptide by fusing the last nine residues of SAPK3/p38γ 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/p38γ 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/p38γ (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/p38γ nor SAPK2a/p38α 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 SAPK2a/p38α 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 that SAPK3/p38γ is responsible for the phosphorylation of SAP90 at Ser^{290} 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

© 2004 Biochemical Society

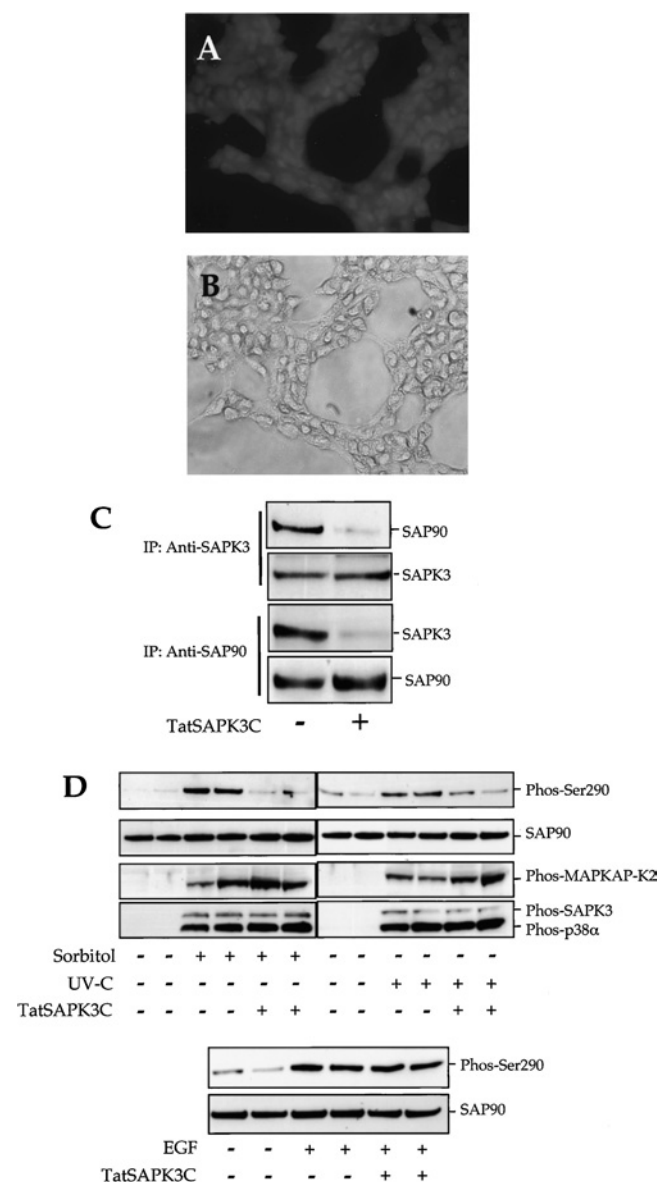

Figure 10 Inhibition of the *in vivo* stress-induced phosphorylation of SAP90 by a peptide corresponding to the C-terminal residues of SAPK3/p38γ

(**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/p38γ 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 μM TatSAPK3C peptide, then exposed for 15 min to 0.5 M sorbitol, 100 ng/ml EGF or UV-C radiation (200 J/m^2), followed by a 30 min incubation. SAP90 was precipitated with GSH–Sepharose from aliquots of the extracts (20 μg of protein), denatured, electrophoresed and immunoblotted as described in the legend to Figure 7. To examine SAPK activation, 50 μg of cell lysates were immunoblotted with an antibody that recognizes MAPKAP-K2 phosphorylated at Thr^{334} or with the SAPK2a/p38α phospho-specific antibody recognizing phosphorylated SAPK3/p38γ (see the Materials and methods section).

and SAPK3/p38γ, which phosphorylate SAP90 at Thr^{287} and Ser^{290}. Furthermore, we also show that SAP90 is phosphorylated *in vivo* at Thr^{287} and that Ser^{290} 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 Ser^{290} 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 Ser^{290} in response to osmotic shock or UV-C radiation. The time course of Ser^{290} phosphorylation paralleled the activation of SAPK3/p38γ 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/p38α and SAPK2b/p38β 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 Ser^{290}, 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 μM) 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/p38δ 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/p38γ is a strong candidate kinase for phosphorylating SAP90 at Ser^{290} under stressful conditions, given that its activation was similar to Ser^{290} phosphorylation and its activity is not inhibited by SB 203580 and PD 184352. Moreover, we also show that the phosphorylation of SAP90 by SAPK3/p38γ, but not by ERK2 or SAPK4/p38δ, is blocked by preincubation of SAP90 with synthetic peptides corresponding to the C-terminal six or eight residues of SAPK3/p38γ. 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/p38α or ERK1/ERK2 activity. These results strongly suggest that SAPK3/p38γ is the stress-activated kinase that phosphorylates SAP90 at Ser^{290}.

In a previous study, we reported that SAPK3/p38γ is the only MAPK family member that is capable of binding to the PDZ domain of α1-syntrophin through its C-terminal sequence (-ETXL) [11]. In the present study, we show that SAPK3/p38γ associates with SAP90 in brain extracts and in cultured cells. Furthermore, SAP90 and SAPK3/p38γ 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/p38γ binds preferentially to the PDZ domain 3 of SAP90. The C-terminal residue of SAPK3/p38γ 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/p38γ depends on binding to the PDZ domain of SAP90. The C-terminal sequence of SAPK3/p38γ 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/p38γ-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

© 2004 Biochemical Society

the molecular activities of phototransduction [38–41]. Moreover, the C-terminal sequence of PKCα 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 PKCα 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 Ser^{290} 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 β2-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/p38γ phosphorylates residues in the N-terminal half of SAP90, we evaluated whether the activation of SAPK3/p38γ 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/p38γ functionally affects SAP90 and its binding partners.

Results of the present study demonstrate that SAP90 is a novel binding partner for SAPK3/p38γ and a novel *in vivo* substrate for SAPK3/p38γ and ERK1/ERK2. They suggest that phosphorylation of SAP90, and/or binding to SAPK3/p38γ, may play a role in regulating protein–protein interactions at the synapse, in response to stresses or mitogen-related signalling events.

We are grateful to Dr A. Mora for helping in the preparation of cerebellar granule cells and for discussion of the experiments. We thank P. Cohen and C. MacKintosh for helpful advice, J. Leitch and M. Aoubala for preparation of antibodies (Division of Signal Transduction Therapy, University of Dundee), F. B. Caudwell and N. Morrice for amino acid sequencing, M. Peggie for the GST-SAP90 DNA and R. Grier for help in culturing the cells. A.C. and M.G. were supported by U.K. Medical Research Council. G.S. was supported by a doctoral fellowship from the Spanish government (Ministerio de Educacion y Cultura y Deporte) and an EMBO short-term fellowship.

REFERENCES

1 Cohen, P. (1997) The search for physiological substrate of MAP and SAP kinases in mammalian cells. Trends Cell Biol. **7**, 353–361

2 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. **351**, 95–105

3 Kyriakis, J. M. and Avruch, J. (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. **81**, 807–869

4 Goedert, M., Cuenda, A., Craxton, M., Jakes, R. and Cohen, P. (1997) Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. EMBO J. **16**, 3563–3571

5 Eyers, P. A., Craxton, M., Morrice, N., Cohen, P. and Goedert, M. (1998) Conversion of SB 203580-insensitive MAP kinase family members to drug-sensitive forms by a single amino-acid substitution. Chem. Biol. **5**, 321–328

6 Parker, C. G., Hunt, J., Diener, K., McGinley, M., Soriano, B., Keesler, G. A., Bray, J., Yao, Z., Wang, X. S., Kohno, T. et al. (1998) Identification of stathmin as a novel substrate for p38δ. Biochem. Biophys. Res. Commun. **249**, 791–796

7 Knebel, A., Morrice, N. and Cohen, P. (2001) A novel method to identify protein kinase substrates: eEF2 kinase is phosphorylated and inhibited by SAPK4/p38δ. EMBO J. **20**, 4360–4369

8 Knebel, A., Haydon, C. E., Morrice, N. and Cohen, P. (2002) Stress-induced regulation of eukaryotic elongation factor 2 kinase by SB 203580-sensitive and -insensitive pathways. Biochem. J. **367**, 525–532

9 Cuenda, A., Cohen, P., Buee-Scherrer, V. and Goedert, M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). EMBO J. **16**, 295–305

10 Goedert, M., Hasegawa, M., Jakes, R., Lawler, S., Cuenda, A. and Cohen, P. (1997) Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases. FEBS Lett. **409**, 57–62

11 Hasegawa, M., Cuenda, A., Spillantini, M. G., Thomas, G. M., Buee-Scherrer, V., Cohen, P. and Goedert, M. (1999) Stress-activated protein kinase-3 interacts with the PDZ domain of α1-syntrophin. A mechanism for specific substrate recognition. J. Biol. Chem. **274**, 12626–12631

12 Buée-Scherrer, V. and Goedert, M. (2002) Phosphorylation of microtubule-associated protein tau by stress-activated protein kinases in intact cells. FEBS Lett. **515**, 151–154

13 Mertens, S., Craxton, M. and Goedert, M. (1996) SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases. FEBS Lett. **383**, 273–276

14 Lechner, C., Zahalka, M. A., Giot, J. F., Moller, N. P. and Ullrich, A. (1996) ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. Proc. Natl. Acad. Sci. U.S.A. **93**, 4355–4359

15 Cohen, N. A., Brenman, J. E., Snyder, S. H. and Bredt, D. S. (1996) Binding of the inward rectifier K^+ channel Kir 2.3 to PSD-95 is regulated by protein kinase A phosphorylation. Neuron **17**, 759–767

16 Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M. and Cantley, L. C. (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science **275**, 73–77

17 Sheng, M. and Pak, D. T. (2000) Ligand-gated ion channel interactions with cytoskeletal and signaling proteins. Annu. Rev. Physiol. **62**, 755–778

18 Garner, C. C., Nash, J. and Huganir, R. L. (2000) PDZ domains in synapse assembly and signalling. Trends Cell Biol. **10**, 274–280

19 Sheng, M. and Sala, C. (2001) PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. **24**, 1–29

20 Kornau, H. C., Schenker, L. T., Kennedy, M. B. and Seeburg, P. H. (1995) Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science **269**, 1737–1740

21 Husi, H. and Grant, S. G. (2001) Isolation of 2000-kDa complexes of *N*-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. J. Neurochem. **77**, 281–291

22 Kim, E., Naisbitt, S., Hsueh, Y. P., Rao, A., Rothschild, A., Craig, A. M. and Sheng, M. (1997) GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. J. Cell Biol. **136**, 669–678

23 Kim, J. H., Liao, D., Lau, L. F. and Huganir, R. L. (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron **20**, 683–691

24 Pak, D. T., Yang, S., Rudolph-Correia, S., Kim, E. and Sheng, M. (2001) Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP. Neuron **31**, 289–303

25 Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. I. and Scott, J. D. (2000) Targeting of PKA to glutamate receptors through a MAGUK–AKAP complex. Neuron **27**, 107–119

26 Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D. and Garner, C. C. (1993) SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumor suppressor gene dlg-A. J. Biol. Chem. **268**, 4580–4583

27 Mora, A., Sabio, G., Gonzalez-Polo, R. A., Cuenda, A., Alessi, D. R., Alonso, J. C., Fuentes, J. M., Soler, G. and Centeno, F. (2001) Lithium inhibits caspase 3 activation and dephosphorylation of PKB and GSK3 induced by K^+ deprivation in cerebellar granule cells. J. Neurochem. **78**, 199–206

© 2004 Biochemical Society

28 Zhai, R., Olias, G., Chung, W. J., Lester, R. A., tom Dieck, S., Langnaese, K., Kreutz, M. R., Kindler, S., Gundelfinger, E. D. and Garner, C. C. (2000) Temporal appearance of the presynaptic cytomatrix protein bassoon during synaptogenesis. Mol. Cell. Neurosci. **15**, 417–428
29 Nagy, A. K., Shuster, T. A. and Delgado-Escueta, A. V. (1989) Rat brain synaptosomal ATP: AMP-phosphotransferase activity. J. Neurochem. **53**, 1166–1172
30 Campbell, D. G. and Morrice, N. A. (2002) Identification of protein phosphorylation sites by a combination of mass spectrometry and solid phase Edman sequencing. J. Biomol. Technol. **13**, 119–130
31 Marshall, C. J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell (Cambridge, Mass.) **80**, 179–185
32 Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. **364**, 229–233
33 Schwarze, S. R., Ho, A., Vocero-Akbani, A. and Dowdy, S. F. (1999) *In vivo* protein transduction: delivery of a biologically active protein into the mouse. Science **285**, 1569–1572
34 Aarts, M., Liu, Y., Liu, L., Besshoh, S., Arundine, M., Gurd, J. W., Wang, Y. T., Salter, M. W. and Tymianski, M. (2002) Treatment of ischemic brain damage by perturbing NMDA receptor–PSD-95 protein interactions. Science **298**, 846–850
35 Abe, J., Kusuhara, M., Ulevitch, R. J., Berk, B. C. and Lee, J. D. (1996) Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. J. Biol. Chem. **271**, 16586–16590
36 Mody, N., Leitch, J., Armstrong, C., Dixon, J. and Cohen, P. (2001) Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. FEBS Lett. **502**, 21–24
37 Burnett, P. E., Blackshaw, S., Lai, M. M., Qureshi, I. A., Burnett, A. F., Sabatini, D. M. and Snyder, S. H. (1998) Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton. Proc. Natl. Acad. Sci. U.S.A. **95**, 8351–8356
38 Adamski, F. M., Zhu, M. Y., Bahiraei, F. and Shieh, B. H. (1998) Interaction of eye protein kinase C and INAD in *Drosophila*. Localization of binding domains and electrophysiological characterization of a loss of association in transgenic flies. J. Biol. Chem. **273**, 17713–17719
39 Huber, A., Sander, P., Gobert, A., Bahner, M., Hermann, R. and Paulsen, R. (1996) The transient receptor potential protein (Trp), a putative store-operated Ca^{2+} channel essential for phosphoinositide-mediated photoreception, forms a signaling complex with NorpA, InaC and InaD. EMBO J. **15**, 7036–7045
40 Tsunoda, S., Sierralta, J., Sun, Y., Bodner, R., Suzuki, E., Becker, A., Socolich, M. and Zuker, C. S. (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature (London) **388**, 243–249
41 Xu, X. Z., Choudhury, A., Li, X. and Montell, C. (1998) Coordination of an array of signaling proteins through homo- and heteromeric interactions between PDZ domains and target proteins. J. Cell Biol. **142**, 545–555
42 Staudinger, J., Lu, J. and Olson, E. N. (1997) Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-α. J. Biol. Chem. **272**, 32019–32024
43 Hirbec, H., Francis, J. C., Lauri, S. E., Braithwaite, S. P., Coussen, F., Mulle, C., Dev, K. K., Couthino, V., Meyer, G., Isaac, J. T. et al. (2003) Rapid and differential regulation of AMPA and kainate receptors at hippocampal mossy fibre synapses by PICK1 and GRIP. Neuron **37**, 625–638
44 Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999) A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β2-adrenergic receptor. Nature (London) **401**, 286–290
45 Hall, R. A., Premont, R. T., Chow, C. W., Blitzer, J. T., Pitcher, J. A., Claing, A., Stoffel, R. H., Barak, L. S., Shenolikar, S., Weinman, E. J. et al. (1998) The β2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. Nature (London) **392**, 626–630
46 Matsuda, S., Mikawa, S. and Hirai, H. (1999) Phosphorylation of serine-880 in GluR2 by protein kinase C prevents its C terminus from binding with glutamate receptor-interacting protein. J. Neurochem. **73**, 1765–1768
47 Chung, H. J., Xia, J., Scannevin, R. H., Zhang, X. and Huganir, R. L. (2000) Phosphorylation of the AMPA receptor subunit GluR2 differentially regulates its interaction with PDZ domain-containing proteins. J. Neurosci. **20**, 7258–7267
48 Kim, E., Niethammer, M., Rothschild, A., Jan, Y. N. and Sheng, M. (1995) Clustering of shaker-type K^+ channels by interaction with a family of membrane associated guanylate kinases. Nature (London) **378**, 85–88
49 Hsueh, Y. P., Kim, E. and Sheng, M. (1997) Disulfide-linked head to head multimerization in the mechanism of ion channel clustering by PSD-95. Neuron **18**, 803–814

Received 27 October 2003/7 January 2004; accepted 23 January 2004
Published as BJ Immediate Publication 23 January 2004, DOI 10.1042/BJ20031628

© 2004 Biochemical Society