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Phosphorylation of the mitochondrial protein Sab by stress-activated protein kinase 3☆

Naomi W. Court,[a] Ivana Kuo,[a] Oonagh Quigley,[a] and Marie A. Bogoyevitch[a,b,*]

[a] *Cell Signalling Laboratory, Biochemistry and Molecular Biology, University of Western Australia, Crawley, Western Australia 6009, Australia*
[b] *Western Australian Institute for Medical Research, Perth 6000, Australia*

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Abstract

Mitogen-activated protein kinases (MAPKs) transduce extracellular signals into responses such as growth, differentiation, and death through their phosphorylation of specific substrate proteins. Early studies showed the consensus sequence (Pro/X)-X-(*Ser*/*Thr*)-Pro to be phosphorylated by MAPKs. Docking domains such as the "kinase interaction motif" (KIM) also appear to be crucial for efficient substrate phosphorylation. Here, we show that stress-activated protein kinase-3 (SAPK3), a p38 MAPK subfamily member, localizes to the mitochondria. Activated SAPK3 phosphorylates the mitochondrial protein Sab, an in vitro substrate of c-Jun N-terminal kinase (JNK). Sab phosphorylation by SAPK3 was dependent on the most N-terminal KIM (KIM1) of Sab and occurred primarily on Ser321. This appeared to be dependent on the position of Ser321 within Sab and the sequence immediately surrounding it. Our results suggest that SAPK3 and JNK may share a common target at the mitochondria and provide new insights into the substrate recognition by SAPK3.
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Keywords: Stress-activated protein kinase-3; Sab; Kinase interaction motif; Mitochondria; Substrate recognition

Signal transduction pathways integrate a diversity of extracellular signals to allow complex biological responses such as growth, differentiation, and death. The proline-directed serine/threonine protein kinases known as the mitogen-activated protein kinases (MAPKs) mediate nuclear, transcriptional events through their phosphorylation of specific transcription factors [1]. However, MAPK substrates have been increasingly found in compartments such as the cytoskeleton [2], plasma membrane [3] or mitochondria [4].

A variety of protein domains are important for the specificity of interactions between signaling proteins [5]. Furthermore, conserved regions called "docking domains" or "kinase interaction motifs" (KIMs) mediate interactions of phosphatases, MAPK kinases, scaffolds and substrates with specific MAPKs [4,6,7]. For MAPK substrates, this interaction is critical for their phosphorylation [4]. Another determinant of substrate phosphorylation is the sequence immediately surrounding the Ser/Thr to be phosphorylated. For the archetypical MAPK, extracellular signal-regulated kinase (ERK), this is (Pro/X)-X-(*Ser*/*Thr*)-Pro [8]. More recent work has defined more specific consensus sequences based on how amino acids within the kinase active site make contact with the substrate phosphorylation site [9].

The MAPK family has expanded following the discovery of MAPKs activated in response to stresses such as osmotic shock, hypoxia, and ionizing radiation. These are the "stress-activated protein kinases" (SAPKs), and include the c-Jun N-terminal (JNK) MAPK family [10,11], and the p38 MAPK members p38α, p38β, p38γ, and p38δ (SAPK2a, SAPK2b, SAPK3, and SAPK4, respectively) [12]. Whereas the

☆ *Abbreviations:* MAPK, mitogen-activated protein kinase; KIM, kinase interaction motif; ERK, extracellular signal-regulated kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; RT, room temperature; GST, glutathione *S*-transferase; GSH, glutathione; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +61-8-6488-1148.
E-mail address: marieb@cyllene.uwa.edu.au (M.A. Bogoyevitch).

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study of the biological functions of SAPK2a/b has been facilitated by the use of chemical inhibitors [13], the actions of SAPK3 and SAPK4 remain relatively poorly defined. For example, studies on SAPK3 have primarily focused on its regulation following overexpression [14]. Possible substrates for SAPK3 have included the dystrophin complex protein α1-syntrophin [3] and the microtubule-associated protein tau [2]. Here, we report that SAPK3 localizes to the mitochondria of cardiac myocytes and HEK293 fibroblasts. We also show that SAPK3 phosphorylates Sab, a mitochondrial protein phosphorylated by JNK. This phosphorylation is dependent on the most N-terminal KIM of Sab, and Ser321 is the major site of phosphorylation by both SAPK3 and JNK2. This suggests that SAPK3 and JNK may share a common target at the mitochondria. Furthermore, using site-directed mutagenesis, we have identified some of the sequence determinants responsible for the favoring of Ser321 by SAPK3 within the Sab protein.

Experimental methods

Antibodies and inhibitors. The anti-SAPK3 rat monoclonal antibody was produced as described previously [15]. The anti-HSP60 mouse monoclonal antibody was purchased from Stress-Gen. Hoechst 33258 was from Sigma. Mitotracker Red, Alexa-488, and Alexa-546 conjugated antibodies were from Molecular Probes. The peptide based on the C-terminus of SAPK3 (Biotin-Aminohexanoic acid-Arg-Gln-Leu-Gly-Ala-Arg-Val-Pro-Lys-Glu-Thr-Ala-Leu-OH) was synthesized by Proteomics International.

Cell culture and immunostaining. Cardiac ventricular myocytes were isolated from 1-day-old rats [16]. These cells and HEK293 fibroblasts were cultured on glass coverslips [15].

Cells were treated with Mitotracker Red (300 nM, 40 min, 37 °C) or left untreated, then fixed, permeabilized, and blocked [15]. Cells were then stained with the SAPK3 antibody (6.6 μg/ml), or the HSP60 antibody (20 μg/ml) (60 min, 37 °C), followed by Alexa conjugated secondary antibodies (6.6 μg/ml, 60 min, 37 °C). Nuclei were stained with Hoechst 33258 (2 μg/ml in 5 mM Tris–HCl, 1 mM EDTA; pH 7.4; 5 min, RT). Stained cells were viewed by scanning confocal microscopy [15].

Site-directed mutagenesis. We used wild-type GST-Sab(219–425) and KIM mutant Sab constructs [4]. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to alter wild-type Sab. Specifically, wild-type Sab has four possible MAPK phosphorylation sites, Ser301 ($_{298}$GPT*S*PSE$_{304}$), Ser321 ($_{318}$DLP*S*PVS$_{324}$), Ser346 ($_{343}$GAS*S*PEC$_{349}$), and Ser391 ($_{388}$SST*S*PEG$_{394}$) that conform to the consensus sequence (Pro/X)-X-(*Ser*/*Thr*)-Pro. We created the following changes around Ser321 or Ser391 as indicated by the underlining:

$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$SST*S*PEG$_{324}$ (the 321/391 mutant),
$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$DLP*S*PES$_{324}$ (the V323E mutant),
$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$DLP*S*PAS$_{324}$ (the V323A mutant),
$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$DLP*S*PVE$_{324}$ (the S324E mutant),
$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$DLP*S*PVA$_{324}$ (the S324A mutant),
$_{318}$DLP*S*PVS$_{324}$ mutated to $_{318}$DLPAPVS$_{324}$ (the S321A mutant),
$_{388}$SST*S*PEG$_{394}$ to $_{388}$SSTAPEG$_{394}$ (the S391A mutant), and
$_{388}$SST*S*PEG$_{394}$ to $_{388}$DLP*S*PVS$_{394}$ (the 391/321 mutant).

With the exception of the 391/321 mutant, changes were introduced into the wild-type GST-Sab sequence. For the 391/321 mutant, we made changes in the S321A mutant. All mutations were confirmed by sequencing and then the GST-fusion proteins were prepared.

Activation of GST-SAPK3, GST-JNK2, and GST-ERK2 in vitro. The following proteins were prepared as fusions to GST: SAPK3, ERK2, JNK2, c-Jun(1–135), ATF2(19–96), MEK1(EE), MKK4(ED), Sab(219–425), and α1-syntrophin. MKK6(DD) was expressed as a fusion to maltose-binding protein.

Each MAPK (80 μg/ml) was incubated with its upstream kinase (160 μg/ml) in activation buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, and 20 mM β-glycerophosphate; pH 7.5) containing 330 μM ATP, overnight at 30 °C. The specific combinations were GST-SAPK3 with MalE-MKK6(DD), GST-ERK2 with GST-MEK1(EE), and GST-JNK2 with GST-MKK4(ED). The following day, GSH–Sepharose in 10 mM EDTA, 50 mM Tris–HCl, pH 7.5, was added. The captured GST-fusion proteins were eluted with reduced GSH and then dialyzed against activation buffer.

Protein kinase assays. The SAPK3 C-terminal peptide (10 or 100 μM) was preincubated with the substrate for 10 min at 30 °C. Each activated protein kinase (1 μg) was then incubated with substrate (10 μg) in activation buffer containing 10 μM ATP and 1 μCi [γ-^{32}P]ATP at 30 °C for 35 min. The reaction components were separated by SDS–PAGE. Following autoradiography, the protein bands of interest were excised and ^{32}P incorporation was quantitated using Cerenkov counting. One way analysis of variance was used to analyze the results of kinase assays, with the Statview SE + Graphics program. Values of $p < 0.01$ were considered significant.

Results

SAPK3 co-localizes with the mitochondria of cells

The accessibility of a protein kinase to a substrate can control intracellular phosphorylation reactions. We have previously shown SAPK3 staining as punctate [15], so as to determine whether this indicated mitochondrial co-localization, we treated cardiac myocytes and HEK 293 fibroblasts with Mitotracker Red, or an antibody against the mitochondrial protein HSP60. We then co-stained with our specific SAPK3 antibody [15]. As shown in Fig. 1, punctate staining was observed with the SAPK3 antibody (panels A, D, G, and J), Mitotracker (panels B and H) or the anti-HSP60 antibody (panels E and K). Overlaying these images showed SAPK3 co-localizing with Mitotracker or HSP60 staining in cardiac myocytes and HEK293 fibroblasts (panels C, F, I, and L). No staining was visualized when the primary antibodies were omitted ([15] and results not shown).

The mitochondrial protein Sab is an in vitro substrate of SAPK3 and loss of the first KIM of Sab prevents phosphorylation

We activated recombinant SAPK3 in vitro with MKK6(DD) (results not shown) and tested its activity towards GST-Sab, GST-ATF2, GST-α1-syntrophin, and GST-c-Jun. We found α1-syntrophin and Sab to be good substrates for SAPK3, ATF2 was less favored, and c-Jun was not a substrate for SAPK3 (Fig. 2A). To determine whether members of the JNK subfamily might have substrate preferences similar to those of

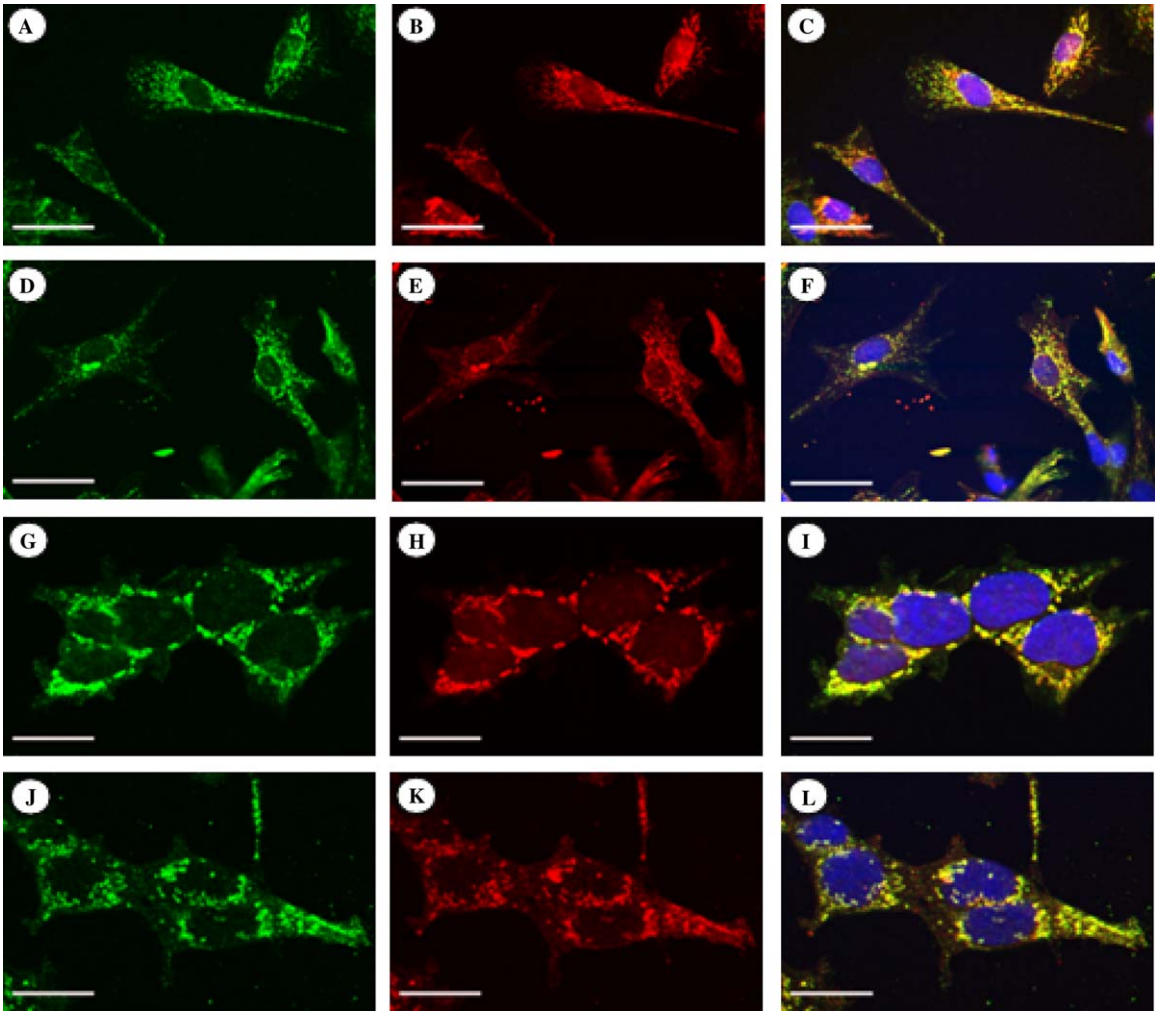

Fig. 1. SAPK3 is localized to the mitochondria. (A–F) Cardiac myocytes were isolated from 1-day-old Sprague–Dawley rats and maintained in culture for 2 days. Cells were then treated with Mitotracker Red (B) or left untreated, then fixed, permeabilized, and stained with anti-SAPK3 (A,D). Cells not treated with Mitotracker were then stained with anti-HSP60 (E). (C,F) The overlay of (A,B), or (D,E), respectively. Yellow staining represents where SAPK3 (green) and mitochondrial markers (red) co-localize. Hoechst 33258 staining (blue) is also included to indicate the position of nuclei in the cells. Scale bar = 36 μm. (G–L) HEK293 fibroblasts were seeded into dishes and cultured for 2 days. Cells were treated with Mitotracker Red (H) or left untreated, then fixed, permeabilized, and stained with anti-SAPK3 (G,J). Cells not treated with Mitotracker were then stained with anti-HSP60 (K). (I,L) The overlay of (G,H), or (J,K), respectively. Yellow staining represents where SAPK3 (green) and mitochondrial markers (red) co-localize. Hoechst 33258 staining (blue) is also included to indicate the position of nuclei in the cells. Scale bar = 18 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

SAPK3, we performed a second series of in vitro kinase assays with activated JNK2. In contrast to SAPK3, JNK2 phosphorylated c-Jun to the greatest extent, followed by Sab. ATF2 was less favored, and α1-syntrophin was a poor substrate (Fig. 2B). These results show that although SAPK3 and JNK share Sab as a substrate, they have differing preferences for other substrates. In contrast, ERK2, a MAPK preferentially activated by growth factors and other mitogenic stimuli, did not phosphorylate Sab, but demonstrated high activity towards a previously characterized substrate, Elk-1 [17] (results not shown).

To determine the mechanism for interaction of SAPK3 with Sab, we used a peptide representing the last 13 amino acids of the rat SAPK3 sequence, Arg-Gln-Leu-Gly-Ala-Arg-Val-Pro-Lys-Glu-Thr-Ala-Leu-OH. This C-terminal tail is crucial for SAPK3 interaction with the PDZ domain of α1-syntrophin and subsequent phosphorylation of this protein [3]. When we incubated activated SAPK3 with the C-terminal peptide (10 or 100 μM final concentration), phosphorylation of α1-syntrophin by SAPK3 was inhibited by $43 \pm 0.9\%$ or $82.5 \pm 1\%$, respectively (Fig. 3). There was no significant effect on the phosphorylation of Sab, with phosphorylation remaining at $100 \pm 8\%$ or $100 \pm 2\%$ in the presence of 10 or 100 μM peptide, respectively. This suggested that SAPK3 uses different mechanisms to recognize Sab and α1-syntrophin.

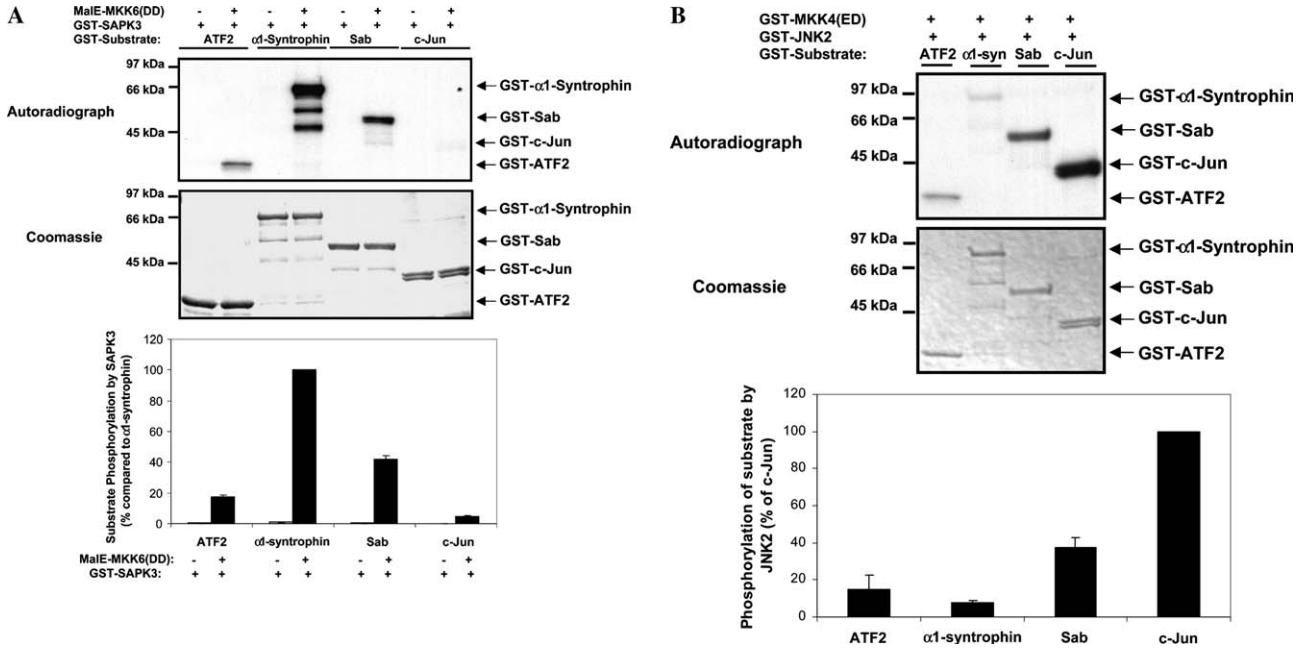

Fig. 2. Sab is a substrate of SAPK3 in vitro. (A) Active GST-SAPK3 (pre-incubated with MalE-MKK6DD) or inactive GST-SAPK3 (pre-incubated with ATP only) was subjected to kinase assays with GST-ATF2(19–96), GST-α1-syntrophin, GST-Sab(219–425) or GST-c-Jun(1–135) as described in Materials and methods. An autoradiograph of the gel is shown in the top panel, the middle panel shows the Coomassie stained gel to illustrate even protein loading, and the lower panel shows the quantitation of three assays as determined by Cerenkov counting of the protein substrate bands. The results are shown as means ± SE. (B) GST-JNK was activated by MKK4(ED) and then subjected to kinase assays with GST-ATF2(19–96), GST-α1-syntrophin, GST-Sab(219–425) or GST-c-Jun(1–135), as described in Materials and methods. An autoradiograph of the gel is shown in the top panel, the middle panel shows the Coomassie stained gel to illustrate even protein loading, and the lower panel shows the quantitation of 4 assays as determined by Cerenkov counting of the protein substrate bands. The results are shown as means ± SE.

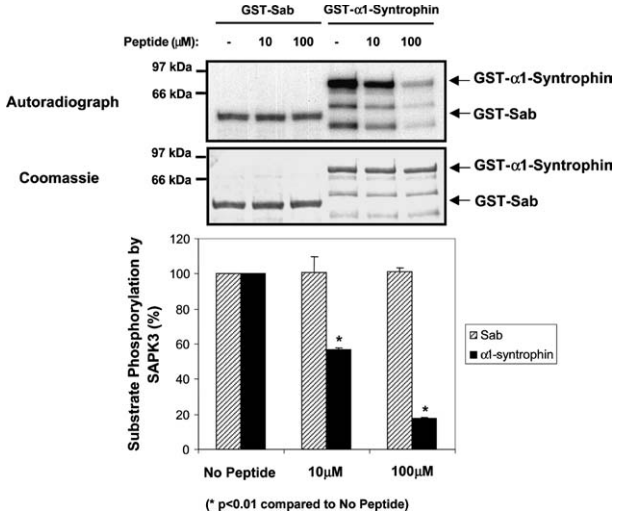

Fig. 3. A SAPK3 C-terminal peptide prevents the phosphorylation of α1-syntrophin, but not Sab, by SAPK3. GST-Sab(219–425) and GST-syntrophin were pre-incubated with the SAPK3 C-terminal peptide and then subjected to kinase assays with activated GST-SAPK3, as described in Materials and methods. An autoradiograph of the gel is shown in the top panel, the middle panel shows the Coomassie stained gel to illustrate even protein loading, and the lower panel shows the quantitation of three assays as determined by Cerenkov counting of the GST-Sab and GST-α1-syntrophin bands. The results are shown as means ± SE.

An alternative interaction motif, the KIM with a consensus R/K(X)$_{3-5}$LXL [18], can mediate interactions in MAPK pathways. The human Sab sequence has KIMs at positions 313–319 (KIM1; **R**PGS**L**D**L**), and 400–406 (KIM2; **R**MKQ**L**S**L**) [4], where the bold residues conform to the KIM consensus. Each KIM has been selectively mutated to show that JNK interacts with Sab using KIM1 [4]. We subjected the -KIM1, -KIM2, and -KIM1/2 mutants of Sab to in vitro kinase assays with activated SAPK3. Phosphorylation of Sab by SAPK3 was reduced by 67.5 ± 0.8% ($p < 0.01$ compared to wild-type Sab) when KIM1 was mutated. There was little effect when KIM2 was mutated, with phosphorylation being reduced by 6 ± 1.5% ($p < 0.01$ compared to wild-type Sab). This indicates a requirement for KIM1 for Sab phosphorylation by SAPK3.

Serine 321 in Sab is the major site of phosphorylation by SAPK3 and JNK2

Wild-type Sab has four possible MAPK phosphorylation sites as shown:

Site 1: Ser301 ($_{298}$GPT*S*PSE$_{304}$),

Site 2: Ser321 ($_{318}$DLP*S*PVS$_{324}$),

Site 3: Ser346 ($_{343}$GAS*S*PEC$_{349}$), and
Site 4: Ser391 ($_{388}$SST*S*PEG$_{394}$).

We used the Predikin program [9] to predict which of these sites could be phosphorylated by SAPK3. Predikin uses structural information on kinase–substrate interactions to more closely define a consensus site of phosphorylation by a serine/threonine protein kinase [9]. Using this program, we predicted the optimal consensus sequence for phosphorylation by SAPK3 was:

$(X)_{-3}$ $(P/V/A/L/S)_{-2}$ $(F/M/L)_{-1}$ $(S/T)_0$ P_{+1} $(F/Xa)_{+2}$ $(R/K/Q/S/L)_{+3}$, where X is any amino acid and Xa is any aliphatic amino acid.

This suggested Ser321 as the most likely site for phosphorylation by SAPK3 with six of the seven amino acids agreeing with the Predikin prediction. We therefore mutated Ser321 (S321A mutant). As a "control" mutation, we mutated Ser391 (S391A mutant).

Phosphorylation of the S321A mutant was greatly reduced, suggesting Ser321 as the major site of phosphorylation (Fig. 4A). Phosphorylation of the S391A mutant was only marginally decreased, suggesting lower phosphorylation of Ser391 by SAPK3. When these Sab mutants were incubated with activated JNK2 we obtained similar results (Fig. 4B). However the phosphorylation of the S391A mutant did not change, suggesting that JNK2 does not phosphorylate Sab on this site. Therefore, Ser321 appears to be the major phosphorylation site in Sab for both SAPK3 and JNK2.

Surrounding sequence composition and location dictate the favoring of Ser321 in Sab

To determine how Ser321 phosphorylation was favored by SAPK3, we conducted a series of mutations around this site. First, to assess whether the position of Ser321 within the Sab protein, or the sequence surrounding Ser321, was favored by SAPK3, we introduced mutations into wild-type Sab or the S321A Sab mutant, to "swap" the sequences around Ser321 and Ser391. This created the 321/391 mutant (with a site surrounding Ser321 changed to the sequence surrounding the original Ser391) or the 391/321 mutant (with a site surrounding Ser391 changed to the sequence surrounding the original Ser321). In the latter case of the 391/321 mutant, this was created in a Sab S321A mutant so that SAPK3 would no longer be able to phosphorylate the Ser321 in its wild-type position, and therefore would not interfere with the interpretation of the results of this analysis.

As shown in Fig. 5, the 321/391 mutant reduced the phosphorylation of Ser321 to approximately 40% of the level of wild-type phosphorylation. The 391/321 mutant showed similar though significantly higher phosphorylation levels than the S321A Sab mutant. This result with the 391/321 mutant suggests that the position of the phosphorylation site within the Sab sequence is a dominant contributor to its ability to be phosphorylated by SAPK3. However, a closer examination of the Sab

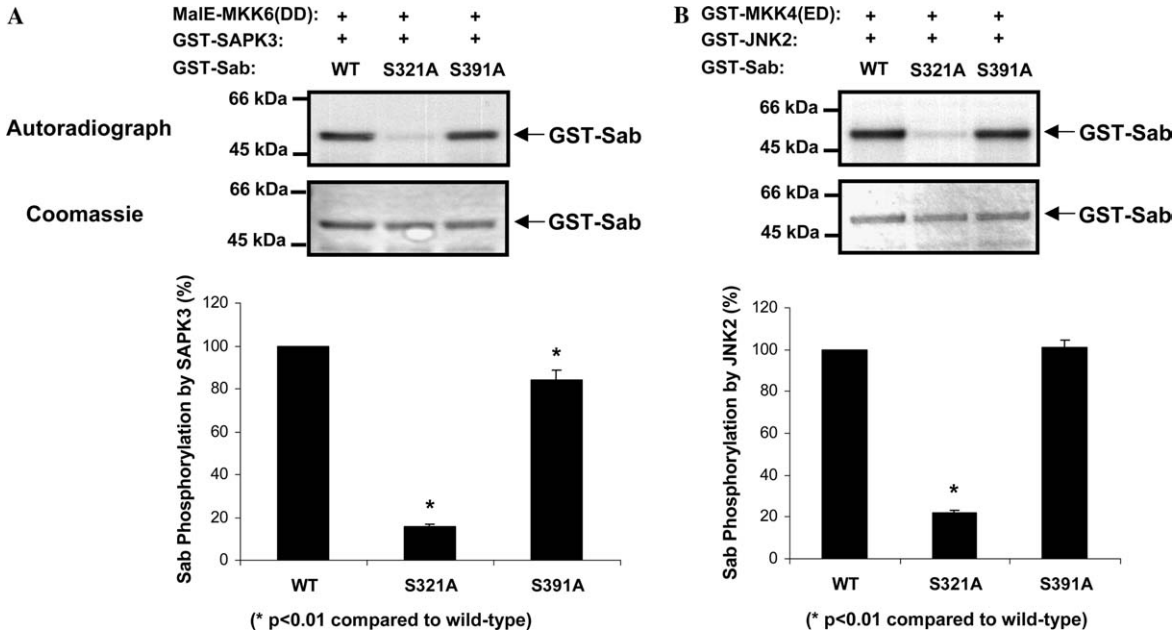

Fig. 4. A Ser321Ala mutant of Sab is not phosphorylated by SAPK3 or JNK2. GST-SAPK3 (A) or GST-JNK2 (B) was activated by MalE-MKK6(DD) or GST-MKK4(ED), respectively, and subjected to kinase assays with wild-type GST-Sab(219–425) or mutants where serine has been changed to alanine at position 321 (S321A) or 391 (S391A), as described in Materials and methods. Autoradiographs of the gels are shown in the top panels, the middle panels show the Coomassie stained gels to illustrate even protein loading, and the lower panels show the quantitation of 4 assays as determined by Cerenkov counting of the GST-Sab bands. The results are shown as means ± SE.

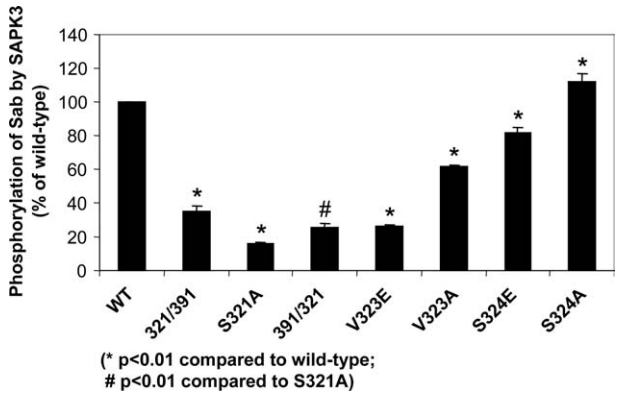

Fig. 5. The phosphorylation of Sab by SAPK3 is dependent on the surrounding sequence and the position of the site within Sab. GST-SAPK3 was activated by MalE-MKK6(DD) and subjected to kinase assays with wild-type GST-Sab(219–425), GST-Sab(321/391) mutant, GST-Sab S321A mutant, GST-Sab(391/321) mutant, GST-Sab V323E mutant, GST-Sab V323A mutant, GST-Sab S324E mutant, or GST-Sab S324A mutant as described in Materials and methods. The graph shows the quantitation of 3 assays as determined by Cerenkov counting of the GST-Sab bands. The results are shown as means ± SE.

sequence shows that the residue at the −2 position of the Ser321 site is the second essential hydrophobic residue in KIM1 as shown in bold in the following sequence $_{313}$**R**PG**S**L**D**L**P***S*PV**S**$_{324}$. Thus, the 321/391 mutant may no longer be phosphorylated only because the integrity of KIM1 has been disrupted. To evaluate this further, mutations were introduced to only change Val323 (i.e., the +2 position), thus creating V323E and V323A mutants, or to change only Ser324 (i.e., the +3 position), thus creating S324E and S324A mutants. Changing these amino acids to Glu (E) more closely resembled the sequences surrounding the other putative MAPK consensus sites in Sab. The replacement with Ala (A) further defined the sequence requirements at the +2 and +3 position of this site.

The V323E mutant decreased phosphorylation of Sab by SAPK3 by approximately 75% (Fig. 5). Introduction of Ala at this position also affected phosphorylation, albeit to a lesser extent. The S324E mutant only decreased the phosphorylation of Sab by SAPK3 by approximately 15%, and the introduction of Ala here improved the phosphorylation of Sab by approximately 10%. Collectively, these results suggest that both the position and the exact sequence surrounding the phosphorylation site within the target protein are important in the phosphorylation of Sab by SAPK3.

Discussion

When compared to the archetypical p38 MAPKs, SAPK2a (p38α), and SAPK2b (p38β), SAPK3 is a relatively poorly studied member of the p38 MAPK family. In this study, we have shown that endogenously expressed SAPK3 co-localizes with the mitochondria of both cardiac myocytes and HEK293 fibroblasts. In this regard, parallels can be drawn with the JNK MAPKs that have also been shown to act at the mitochondria [4,19]. Importantly, this co-localization suggests that mitochondrial substrates for SAPK3 will be identified.

We have shown in this study that activated SAPK3 phosphorylates the mitochondrial protein Sab. This protein, first described as a binding partner of Bruton's tyrosine kinase (Btk) [20,21], is also an in vitro substrate of the JNKs [4]. The sharing of substrates by protein kinases can provide important levels of control in intracellular signaling pathways. Thus, when two or more protein kinases share an identical phosphorylation site in a substrate, this can allow different inputs to result in the same effect. An example of this is seen with the phosphorylation of the Mnk family of protein kinases by ERKs in response to growth factors and SAPK2a in response to stress stimuli. This provides a mechanism for both growth factors and stress stimuli to regulate Mnk activity [22]. Alternatively, different protein kinases might phosphorylate the same substrate but at distinct sites. In some cases this concerted phosphorylation may be required for full substrate activation. In other cases the different phosphorylation events might result in opposite actions. An example of this latter situation is seen when JNK phosphorylates the N-terminal Ser63 and Ser73 of c-Jun to promote c-Jun activity but ERK phosphorylates c-Jun at an inhibitory C-terminal site [23].

Within Sab we have identified serine 321 as the major site of phosphorylation by both SAPK3 and JNK2. This might be expected when we showed that SAPK3 interacts with the same KIM as JNK (KIM1) [4]. Interestingly, SAPK3 but not JNK2 also phosphorylates serine 391, albeit to a much lesser extent that serine 321. Therefore, there are subtle differences, as well as a shared site, when Sab is phosphorylated by JNK2 and SAPK3.

When analyzing the possible phosphorylation sites within a substrate, the definition of consensus sequences has proven useful. For MAPK substrates, the definition of the consensus site (Pro/X)-X-(*Ser*/*Thr*)-Pro has relied on the use of synthetic peptides to define the optimal determinants of phosphorylation [8]. Evaluating the sequences surrounding the known sites of phosphorylation by SAPK3 (e.g., α1-syntrophin: PPAS_{193}PLQ and QPSS_{201}PGP [3]; tau: APKT_{181}PPS, SPGS_{202}PGT, SPGT_{205}PGS, VVRT_{231}PPK, VYKS_{396}PVV, GDTS_{404}PRH, and SNDS_{422}PQL [2]; SAP90/PSD-95: TAMT_{287}PTS and TPTS_{290}PPR [24]) has suggested this consensus also holds true for SAPK3. However, this simple comparison with only a limited number of SAPK3 substrates does not allow the more precise prediction of SAPK3 substrate preferences.

An advance in comes with Predikin [9] which uses structural information derived from the interactions of Ser/Thr protein kinases and their substrates to define

preferred sites for phosphorylation. For SAPK3, Predikin defines the consensus sequence for phosphorylation to be:

$(X)_{-3}$ $(P/V/A/L/S)_{-2}$ $(F/M/L)_{-1}$ $(S/T)_0$ P_{+1} $(F/Xa)_{+2}$ $(R/K/Q/S/L)_{+3}$, where X is any amino acid and Xa is any aliphatic amino acid.

When the sequences surrounding the α1-syntrophin phosphorylation sites Ser193 (PPA*S*$_{193}$PLQ) and Ser201 (QPS*S*$_{201}$PGP) [3] are compared, six out of seven or five out of seven amino acids, as denoted by the underlining, agree with the Predikin prediction. When four of the other putative MAPK phosphorylation sites in α1-syntrophin which are not phosphorylated by SAPK3 are examined (LTV*S*$_{43}$PAD, KDV*S*$_{173}$PYF, GWD*S*$_{189}$PPA, SVE*S*$_{385}$ PQE), only three out of seven or four out of seven amino acids agree with the Predikin prediction in each case, as denoted by the underlining. For the SAPK3 phosphorylation sites in tau, there is reasonable agreement, with most of the sites having five or six out of seven amino acids agreeing with the Predikin prediction. Specifically these are APK*T*$_{181}$PPS, SPG*S*$_{202}$PGT, SPG*T*$_{205}$PGS, VVR*T*$_{231}$PPK, VYK*S*$_{396}$PVV, GDT*S*$_{404}$ PRH, and SND*S*$_{422}$PQL [2], again with the underlining denoting the amino acids agreeing with the Predikin prediction. In addition, there is good agreement between the SAPK3 phosphorylation sites of SAP90 and the Predikin prediction with six out of seven amino acids in agreement for both sites (TAM*T*$_{287}$PTS, TPT*S*$_{290}$PPR [24]). Thus, it will be interesting to evaluate the effectiveness of the Predikin predictions as more substrates of SAPK3 are identified.

Another possible use of the Predikin program is with the comparison of the optimal consensus sequences for SAPK3 and JNK2 phosphorylation. This suggests that these two SAPKs may not always share similar preferences for substrate phosphorylation sites because the predicted optimal substrate recognition sequence for JNK2 differs from that of SAPK3 and is $(L/I/M/F)_{-3}$ $(E/D/S/R)_{-2}$ $(F/M/L)_{-1}$ $(S/T)_0$ P_{+1} $(P/F/L/I/D/E)_{+2}$ $(R/K/Q/S/L)_{+3}$. This is supported by our results showing that SAPK3 and JNK2 have different substrate preferences with regard to α1-syntrophin and c-Jun. Furthermore, under some circumstances active ERK2 also localizes to the mitochondria [25]. The Predikin consensus site for phosphorylation by ERK2 is very similar to that for SAPK3, being $(R/K/F)_{-3}$ $(P/V/A/L/S)_{-2}$ $(F/M/L)_{-1}$ $(S/T)_0$ P_{+1} $(F/XA)_{+2}$ $(R/K/Q/S/L)_{+3}$. This suggests that substrates will be shared by ERK2 and SAPK3. In contrast, we have seen that Sab is phosphorylated by SAPK3 and JNK2, but not ERK2 (results not shown). Therefore, other features must account for these differences in substrate recognition.

The C-terminal sequence of SAPK3, KETXL, is essential for the interaction of SAPK3 with the PDZ domain of α1-syntrophin, and the subsequent phosphorylation of this protein [3]. However, we found that SAPK3 phosphorylates Sab via a mechanism that uses KIM1 of Sab because this substrate does not appear to have a PDZ domain. We are the first to show this phosphorylation of a substrate by SAPK3 requires a KIM motif. This suggests that SAPK3 can, like other kinases, use this mechanism in substrate recognition. When comparing the sequences of Sab to those of other substrates of SAPK3, such as ATF2 [14] and tau [2], KIM-like sequences $(R/K(X)_{3-5}LXL)$ can be identified in both of these substrates. Specifically, $_{47}$**K**KMP**L**D**L**$_{53}$ in ATF2 has been previously implicated as a KIM [26,27], whilst $_{221}$**R**EPKK**V**A**V**$_{228}$ in tau appears to resemble the KIM sequence.

In conclusion, these studies suggest that SAPK3 and JNK2 may share a common target at the mitochondria. Furthermore, we have shown that the sequence immediately surrounding the phosphorylation site of a substrate, as well as the position of that site within the substrate protein are important for recognition by SAPK3.

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