A new c-Jun N-terminal kinase (JNK)-interacting protein, Sab (SH3BP5), associates with mitochondria

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We have identified a novel c-Jun N-terminal kinase (JNK)-interacting protein, Sab, by yeast two-hybrid screening. Sab binds to and serves as a substrate for JNK *in vitro*, and was previously found to interact with the Src homology 3 (SH3) domain of Bruton's tyrosine kinase (Btk). Inspection of the sequence of Sab reveals the presence of two putative mitogen-activated protein kinase interaction motifs (KIMs) similar to that found in the JNK docking domain of the c-Jun transcription factor, and four potential serine–proline JNK phosphorylation sites in the C-terminal half of the molecule. Using deletion and site-directed mutagenesis, we demonstrate that the most N-terminal KIM in Sab is essential for JNK binding, and that, as with c-Jun, physical interaction with JNK is necessary for Sab phosphorylation. Interestingly, confocal immunocytochemistry and cell fractionation studies indicate that Sab is associated with mitochondria, where it co-localizes with a fraction of active JNK. These and previously reported properties of Sab suggest a possible role in targeting JNK to this subcellular compartment and/or mediating cross-talk between the Btk and JNK signal transduction pathways.

Key words: apoptosis, signal transduction, stress kinase.

INTRODUCTION

Vertebrate cells contain multiple mitogen-activated protein (MAP) kinase pathways, of which the three best characterized terminate in the extracellular-signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAP kinases. Individual MAP kinase subfamilies are activated by distinct stimuli; the ERK pathway is stimulated primarily by growth factors and trophic signals, whereas the JNK and p38 pathways are activated most potently by inflammatory cytokines and a variety of chemical and radiant stresses [1]. MAP kinase pathways are thought to play a role in the regulation of a wide variety of cellular processes. The JNK pathway, for example, has been implicated in cell proliferation, differentiation, apoptosis, immune cell function and embryonic morphogenesis [2]. In view of the pleiotropic endpoints of JNK signalling, it seems likely that JNK must act on many cellular substrate proteins; however, only a relatively small number of JNK targets have been identified so far [2].

The most thoroughly characterized JNK substrate is c-Jun, a transcription factor whose activity is selectively stimulated through JNK-mediated phosphorylation of two regulatory serine residues (Ser-63/Ser-73) contained within the N-terminal transcriptional activation domain [3,4]. Efficient modification of Ser-63/Ser-73 in c-Jun depends on binding of JNK to c-Jun via a docking motif located immediately N-terminal to the phosphoacceptor sites themselves (the delta region) [5]. JNK also phosphorylates a number of other transcription factors, including ATF-2 (activating transcription factor-2), Elk-1 and NFAT (nuclear factor of activated T-cells), each of which also contains docking motifs which enable binding of JNK [6]. Protein–protein interactions therefore play a major role in JNK substrate selection.

JNK also participates in docking interactions with a variety of scaffold proteins, upstream activators and MAP kinase phosphatases [2,6]. In most cases the functional consequences of these interactions are not yet fully understood; however, they are likely to play an important role in regulating JNK signal transduction. In particular, the JNK-interacting protein 1 (JIP1), JIP2 and JIP3 [7,8] scaffold proteins organize complexes containing multiple JNK pathway components, including JNK itself, immediate upstream activators such as MKK (MAP kinase kinase) 4/7, and more distal upstream elements such as MLK (mixed-lineage kinase) and MEKK (MAP kinase/ERK kinase kinase) family kinases [2]. Such scaffold complexes are thought to enhance the efficiency and specificity of JNK signalling by minimizing cross-talk with other pathways, and may also play a role in targeting JNK to a specific subcellular localization and/or restricted subset of substrates [2].

Understanding how JNK activation evokes specific and distinct biological responses in different cellular contexts represents a major challenge. Changes in gene transcription induced via c-Jun or other transcriptional effectors represent an important endpoint of JNK signalling; however, it is clear that JNK can also act via non-transcriptional mechanisms. For example, studies using cells from compound mutant mice that lack functional *JNK1* and *JNK2* genes have established that JNK is required for apoptosis in mouse embryo fibroblasts exposed to

Abbreviations used: Btk, Bruton's tyrosine kinase; CEF, chick embryo fibroblasts; CoxIV, cytochrome *c* oxidase complex IV; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; HFF, human foreskin fibroblasts; JIP1 (etc.), JNK-interacting protein 1 (etc.); JNK, c-Jun N-terminal kinase; KIM, kinase interaction motif; MAP kinase, mitogen-activated protein kinase; PDI, protein disulphide isomerase; SH3, Src homology 3; SP residue, serine–proline residue.

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UV irradiation and other stresses [9]. Importantly, in this system JNK triggers cell death by promoting the release of cytochrome *c* from mitochondria via a mechanism that is independent of new gene transcription [9].

Understanding non-transcriptional JNK signalling processes will require a comprehensive identification of non-nuclear JNK substrates and detailed characterization of the mechanisms that target JNK to specific subcellular compartments. In view of the important role that protein–protein interactions play in targeting JNK to known substrates and regulators, we sought to identify novel JNK-interacting proteins by yeast two-hybrid screening. This approach led to the isolation of a protein, Sab (SH3BP5), which we have shown can bind to and serve as a substrate for JNK *in vitro*. Although the biological function of Sab is not yet known, we have found that Sab associates with mitochondria, where it co-localizes with a fraction of active JNK. Interestingly, Sab was previously isolated in a screen for proteins capable of binding to the Src homology 3 (SH3) domain of Bruton's tyrosine kinase (Btk) [10]. Btk is essential for B-cell development [11,12] and has been implicated in both JNK signalling and apoptosis in B cells and mast cells [13]. These observations suggest that Sab could play a role in targeting JNK and Btk to mitochondria and/or mediating cross-talk between the JNK and Btk signalling pathways.

EXPERIMENTAL

Yeast two-hybrid screen

To identify novel JNK-interacting proteins, a two-hybrid screen was carried out using the DupLEX-A (Origene) system. A human HeLa cell cDNA library was screened using the rat homologue of JNK3 [14] (also known as SAPK-β; [15]). Of 1.5×10^6 independent clones screened, 11 were found to show a reproducible interaction with JNK3. The cDNAs contained within these clones were purified and sequenced.

Cell lines and preparation of cell extracts

Normal and Myc-transformed chick embryo fibroblasts (CEF) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) newborn calf serum at 41 °C in a humidified 5% CO_2 incubator, as reported previously [16,17]. Human squamous epithelial carcinoma (A431) cells were grown in DMEM supplemented with 10% (v/v) foetal calf serum at 37 °C in 5% CO_2. Human foreskin fibroblasts (HFF) were grown in DMEM supplemented with 20% (v/v) foetal calf serum under humid conditions at 37 °C in 5% CO_2. Murine mammary epithelial (KIM-2) cells were grown at 37 °C in 5% CO_2 in a 1:1 (v/v) mixture of DMEM and nutrient mixture F12 supplemented with 10% (v/v) foetal calf serum.

For pull-down experiments, cell extracts were prepared from Myc-transformed CEF (150 mm dishes) after treatment with 10 μg/ml anisomycin (Sigma) for 15 min to activate JNK. Myc-transformed CEF were used as a source of active JNK for convenience, owing to their rapid proliferation and high saturation density in culture. After three washes in cold PBS, cells were scraped into 1 ml of Lysis Buffer J, swelled on ice, and extracts were collected after centrifugation, as described previously [18]. Extracts were also prepared for Western blot analysis from normal CEF, A431 cells, HFF and KIM-2 cells without any pretreatment, as above.

Cytosolic and membrane extracts were prepared by centrifugal separation essentially as described in [19]. Briefly, cells were rinsed in PBS prior to resuspension in 5 vol. of Buffer A (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.1 mM PMSF) and lysed gently by incubation in a sonicating water-bath for 5 min, which was followed by two rounds of centrifugation at 750 ***g*** for 10 min to remove nuclei. Supernatants were further centrifuged at 10000 ***g*** for 15 min to generate a pellet fraction enriched in mitochondria. This supernatant was then centrifuged at 100000 ***g*** for 1 h to generate a pellet fraction enriched in endoplasmic and plasma membranes and a residual S-100 cytosol fraction.

DNA manipulations

All routine DNA manipulations were carried out according to standard procedures [20]. Glutathione S-transferase (GST)–Sab-(219–425) fusion protein was generated by cloning the partial cDNA encoding Sab-(219–425) isolated from the yeast two-hybrid screen into pGEX-4T-1 (Amersham Pharmacia Bioscience). C-terminal deletion mutants of Sab-(219–425) were generated by PCR, cloned into the pPCR-Script vector (Stratagene) and their authenticity verified by sequencing. The PCR products for the truncated forms were then cloned into pGEX-4T-1 to generate GST–Sab-(219–390), GST–Sab-(219–353) and GST–Sab-(219–309). Kinase interaction motif (KIM) mutants were generated using the Quik-Change Site Directed Mutagenesis system (Stratagene), according to the manufacturer's instructions. The sequences of the primers used in PCR and site-directed mutagenesis are available on request.

GST pull-down and kinase assays

GST, GST–c-Jun-(1–79) (a gift from I. Morgan, Department of Veterinary Pathology, University of Glasgow, U.K.) and the GST–Sab proteins were expressed and purified from *Escherichia coli* as described previously [21]. For JNK pull-down assays, glutathione–Sepharose beads loaded with 5 μg of GST, GST–c-Jun-(1–79) or GST–Sab protein were incubated with 2 mg of cell extract as described [18]. After extensive washing, bound proteins were eluted and resolved by SDS/PAGE, and active JNK was detected by Western blotting using a polyclonal antibody specific for the dual phosphorylated form of JNK (New England Biolabs).

To capture active JNK for kinase assays, 30 μg of GST or GST–c-Jun-(1–79) fusion protein bound to glutathione–Sepharose beads was incubated with 10 mg of anisomycin-activated Myc-transformed CEF cell extract. After extensive washing, each set of beads was divided equally between five 1.5 ml tubes, 3 μg of the different GST–Sab fusion proteins was added and *in vitro* kinase assays were performed in the presence of 20 μM ATP and 0.11 MBq of [γ-^{32}P]ATP [5]. Reactions were resolved by SDS/PAGE and immobilized on nitrocellulose, after which autoradiography was carried out and active JNK was visualized by Western blotting using a polyclonal antibody specific for the phosphorylated form of JNK (New England Biolabs). Where required, the level of phosphorylation of Sab by JNK was quantified by laser densitometry of the autoradiograph, using a 420oe scanner with Quantity One® software (pdi).

Generation of anti-Sab-(219–425) antibodies and Western blotting

GST–Sab-(219–425) was digested with thrombin, and the purified Sab-(219–425) portion was used as an immunogen to generate a polyclonal antiserum in New Zealand white rabbits. The antiserum was then used for Western blotting, immunoprecipitation and immunocytochemistry. For Western blotting of whole-cell extracts, 50 μg of cell extract was resolved by SDS/PAGE, immobilized on nitrocellulose, and probed with anti-Sab-

(219–425) antiserum diluted 1:5000 in TBST (20 mM Tris/HCl, pH 7.6, 275 mM NaCl and 0.1% Tween 20) containing 5% (w/v) non-fat dry milk.

In vitro transcription/translation and immunoprecipitation

Coupled *in vitro* transcription/translations of pPCR-Script-Sab-(1–425) and the series of pcDNA3 vectors containing cDNAs encoding all ten isoforms of JNK (a gift from R. J. Davis; [14]) were carried out from the T7 promoter using the TnT® Coupled Reticulocyte Lysate System (Promega). GST pull-downs were carried out on a portion of the JNK translation reactions using 5 μg of GST, GST–c-Jun-(1–79) and GST–Sab-(219–425) beads, according to a previously reported method [14]. After washing, bound proteins were eluted and resolved by SDS/PAGE, and the gel was fixed, treated with Amplify (Amersham Pharmacia), dried and exposed to Fuji Super RS film. *In vitro* translated Sab-(1–425) was divided between three 1.5 ml tubes and immunoprecipitated with preimmune serum, anti-Sab-(219–425) and anti-Sab-(1–99) as described previously [22]. Immunoprecipitates were resolved by SDS/PAGE, and the gel was processed and analysed by autoradiography as described above.

Immunocytochemistry

CEF, A431 cells, HFF and KIM-2 cells were grown under the conditions described above on glass coverslips, then fixed and permeabilized as described in [23]. The subcellular localization of mitochondria and endoplasmic reticulum was established using antibodies specific for cytochrome *c* oxidase complex IV (CoxIV; Molecular Probes) and protein disulphide isomerase (PDI; Stressgen) respectively. The localization of Sab and active JNK was visualized using anti-Sab-(219–425) and a phospho-JNK monoclonal antibody (Cell Signalling Technologies) respectively. In each case, Sab was visualized using a Texas Red-conjugated anti-rabbit secondary antibody, while the localization of CoxIV, PDI and phospho-JNK was observed using an FITC-conjugated anti-mouse antibody. Mitochondrial localization was confirmed by staining with Mito Tracker CMXRos (Molecular Probes) according to the manufacturer's instructions. Where required, JNK was activated in CEF by treatment with 10 μg/ml anisomycin for 15 min.

RESULTS

Sab binds to and serves as a substrate for JNK

To identify novel JNK-interacting proteins, a yeast two-hybrid screen was performed using JNK3 as a bait. Of 11 potential JNK-interacting clones isolated, one was found to contain a partial cDNA encoding part of a previously identified SH3-domain-binding protein, Sab [10]. In this clone, in-frame fusion between the *lexA* DNA-binding domain and Sab occurred at nucleotide 718 of the Sab cDNA sequence (GenBank accession number AB005047), resulting in a hybrid LexA protein encoding amino acids 219–425 of Sab (Figure 1A).

To confirm that the Sab C-terminus binds to JNK, a peptide comprising amino acids 219–425 of Sab was expressed as a GST fusion protein and tested for JNK binding in a pull-down assay. Extracts prepared from cells treated with anisomycin to activate JNK were incubated with GST–Sab-(219–425), GST–c-Jun-(1–79) or GST alone, and after extensive washing bound proteins were eluted and analysed by Western blotting using an antibody specific for the phosphorylated, active form of JNK. As shown in Figure 1(B), the results of this experiment confirmed that GST–Sab-(219–425), like GST–c-Jun-(1–79), was able to capture

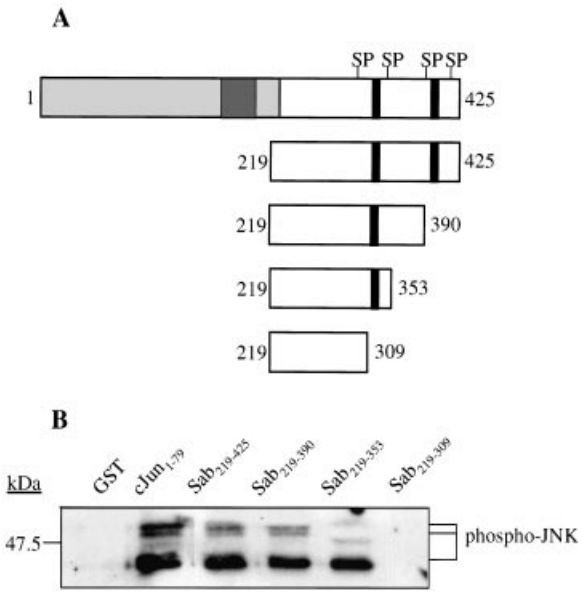

Figure 1 Structural features and interaction of Sab with JNK *in vitro*

(**A**) Diagrammatic representation of structural features of the full-length Sab protein. The N-terminal half of the protein exhibits similarity to the rod domain of myosin and is predicted to have a high probability of forming an extended coiled coil (light shading). Within this region, a segment between amino acids 163 and 193 has been shown previously to bind to the SH3 domain of Btk (dark shading). Four putative SP JNK phosphorylation sites and two potential KIMs [$K/R(X)_{3-5}LXL$; black bars] are located in the C-terminal half of the protein. The relative extent of the JNK3-interacting segment of Sab obtained by two-hybrid screening (amino acids 219–425) is indicated. This segment, and the indicated C-terminally truncated derivatives, were expressed as GST fusion proteins and used in binding assays. (**B**) GST pull-down assays were performed using GST, GST–c-Jun-(1–79) and the panel of GST–Sab proteins indicated in (**A**). Extracts were prepared from cells treated with anisomycin (10 μg/ml) to activate JNK and incubated with the indicated GST fusion proteins. After extensive washing, bound proteins were eluted and analysed by SDS/PAGE followed by Western blotting using an anti-phospho-JNK polyclonal antibody.

active JNK from cell extracts. The 54 kDa isoforms of JNK were less evident when GST–Sab-(219–353) was used as the affinity matrix compared with GST–c-Jun-(1–79), GST–Sab-(219–425) and GST–Sab-(219–390); however, this appears to be due to co-migration with the very abundant GST–Sab-(219–353) fusion protein, which interferes with detection of these higher-molecular-mass species by Western blotting, rather than representing a true difference in the spectrum of JNK isoforms captured by each matrix (see also Figure 2). Preliminary studies indicate that Sab appears to preferentially capture active – as opposed to inactive – JNK (results not shown).

Sab was first isolated by virtue of its ability to bind to the SH3 domain of Btk [10], and the minimal region of Sab required for this interaction was shown to reside between amino acids 163–193 (dark grey box in Figure 1A). Computer analysis of the Sab protein sequence indicates that the N-terminal half of the protein (light grey box in Figure 1A) exhibits similarity to the rod domain of myosin heavy chain, and is predicted to have a high probability of forming an extended coiled coil, while the C-terminal half of Sab, which binds to JNK, exhibits no extended similarity to any other protein in the public databases (G. H. W. May and C. Wiltshire, unpublished work). Within this latter segment, however, are two short sequences that are similar to the KIM [consensus $K/R(X)_{3-5}LXL$, where X stands for any amino acid] which is found in the c-Jun δ domain and other transcription factors that can bind JNK or other MAP kinases [6]. In Sab these

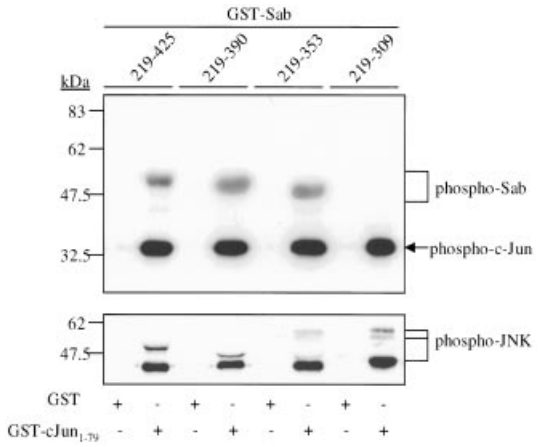

Figure 2 Sab can serve as a substrate for JNK

Active JNK was captured from anisomycin-treated cell extracts using a GST–c-Jun-(1–79) matrix. After extensive washing, the ability of bead-bound JNK to phosphorylate the panel of GST–Sab fusion proteins (Figure 1A) was tested in *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. Kinase reactions were resolved by SDS/PAGE analysis and transferred to nitrocellulose membranes. Phosphorylated proteins were detected by autoradiography (upper panel), while the presence of equal amounts of active JNK in each reaction was confirmed by probing with a phospho-specific JNK antibody (lower panel).

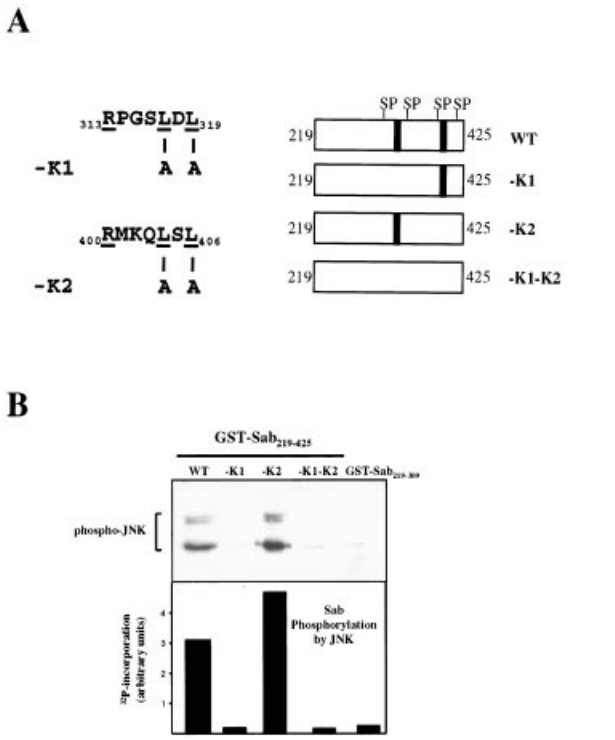

Figure 3 The N-terminal KIM of Sab is essential for JNK binding and phosphorylation activity

(**A**) The N-terminal KIM of Sab is essential for JNK binding. Left panel: each of the putative KIM elements in Sab was eliminated by replacing both leucine residues in the conserved LXL motif with alanine using site-directed mutagenesis. Right panel: KIM mutations were introduced either individually or in combination into GST–Sab-(219–425) to generate mutant derivatives designated -K1, -K2 and -K1-K2. (**B**) Binding of JNK is essential for Sab phosphorylation *in vitro*. Wild-type GST–Sab-(219–425) and the -K1, -K2 and -K1-K2 mutants were used in GST pull-down (upper panel) and JNK kinase assays using GST–Sab-(219–309) for comparison, essentially as described in the legends to Figures 1(B) and 2 respectively. JNK-mediated phosphorylation of GST–Sab proteins was quantified by laser densitometry of the resulting autoradiogram (lower panel).

KIM-like sequences (black boxes in Figure 1A) are located between amino acids 313–319 (RPGSLDL) and 400–406 (RMKQLSL). The C-terminal half of Sab also contains four serine–proline (SP) residues, i.e. Ser^{300}, Ser^{321}, Ser^{346} and Ser^{391} (Figure 1A), which could potentially serve as JNK phosphorylation sites.

To determine whether the putative KIMs in Sab are required for JNK binding, we first prepared C-terminal deletion derivatives of GST–Sab-(219–425) that lacked either one or both sequences (Figure 1A). As shown in Figure 1(B), GST–Sab-(219–390) and GST–Sab-(219–353), which both retain the most N-terminal KIM-like motif, still bind JNK, while GST–Sab-(219–309), which lacks both, does not. These results suggest that at least one putative KIM sequence is required for JNK binding to Sab.

We also investigated whether Sab can serve as a substrate for JNK. To this end, active JNK was captured on a GST–c-Jun-(1–79) matrix [18] and tested for its ability to phosphorylate GST–Sab-(219–425) in the presence of [γ-^{32}P]ATP *in vitro*. Mock reactions using a GST matrix were used to control for non-specific kinase activity. As shown in Figure 2, GST–Sab-(219–425) became labelled when incubated with the GST–c-Jun-(1–79) matrix bearing active JNK (Figure 2, lower panel), but not in the presence of the control matrix, indicating that Sab can be phosphorylated by JNK. Strikingly, although GST–Sab-(219–390) and GST–Sab-(219–353) were also phosphorylated by JNK, GST–Sab-(219–309) was not. The failure of this shortest Sab construct to serve as substrate for JNK could reflect either loss of the site(s) that in longer derivatives is modified by JNK, or, alternatively, that binding of JNK is required for phosphorylation, as in c-Jun [5].

To investigate further the role of the putative Sab KIM elements and the relationship between JNK binding and phosphorylation, site-directed mutagenesis was used to replace both leucines in the conserved LXL motifs with alanine residues (Figure 3A). Analogous mutations in the c-Jun JNK docking region KIM have been shown to disrupt both JNK binding and phosphorylation [24]. Mutant derivatives of GST–Sab-(219–425) lacking one (-K1, -K2) or both (-K1-K2) KIMs were generated and tested for their ability to bind to and serve as substrates for JNK. As shown in Figure 3(B) (upper panel), this analysis revealed that K1 was absolutely required for phospho-JNK binding, whereas K2 was dispensable. Mutants lacking K1 also failed to serve as substrates for JNK when incubated with active JNK bound to a GST–c-Jun-(1–79) matrix (Figure 3B, lower panel). Since these mutants retain all of the potential JNK phosphorylation sites, this demonstrates that binding of JNK is required for efficient phosphorylation of Sab.

Sab interacts with protein kinases encoded by *JNK1*, *JNK2* and *JNK3*

Sab was isolated in a yeast two-hybrid screen using JNK3 as a bait. Since JNK3 has a relatively restricted tissue-specific distribution [25], and antibodies specific for active JNK do not distinguish between products encoded by the different JNK family members, it was of interest to determine whether Sab can also interact with the products of the ubiquitously expressed *JNK1* and *JNK2* genes. To this end, all ten JNK isoforms encoded by the *JNK1*, *JNK2* and *JNK3* genes [14] were synthesized by *in vitro* transcription/translation and tested for

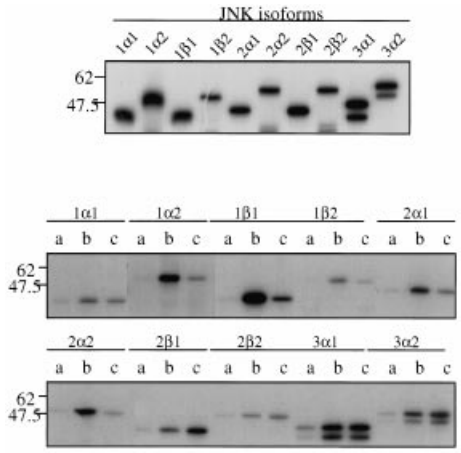

Figure 4 The C-terminal portion of Sab can capture all JNK isoforms

All ten JNK isoforms encoded by the *JNK1*, *JNK2* and *JNK3* genes were synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine. Part of each reaction was subjected to SDS/PAGE and autoradiography to monitor translation (top panel). The remaining portion was incubated with GST (lanes a), GST–c-Jun-(1–79) (lanes b) or GST–Sab-(219–425) (lanes c), and after extensive washing the bound proteins were eluted and analysed by SDS/PAGE and autoradiography (lower panels). Positions of molecular mass markers (kDa) are indicated on the left.

binding to GST–Sab-(219–425) using GST–c-Jun-(1–79) for comparison. Although there was considerable variation in efficiency, GST–Sab-(219–425) was able to bind all JNK isoforms (Figure 4).

Sab is associated with mitochondria

To facilitate immunolocalization and further biochemical studies, a polyclonal rabbit antiserum was raised using the Sab-(219–425) portion of the GST–Sab-(219–425) fusion protein as an immunogen (see the Experimental section for details). The resulting antiserum was tested for its ability to precipitate *in vitro* generated full-length Sab protein using a previously described N-terminal Sab antibody [anti-Sab-(1–99)] for comparison [10]. As shown in Figure 5(A) (left panel), both anti-Sab-(219–425) and anti-Sab-(1–99) precipitated full-length Sab protein translated *in vitro*. When tested in Western blotting, the anti-Sab-(219–425) antibody recognized a protein of approx. 70 kDa in size in human (A431, HFF), murine (KIM-2) and avian (CEF) cells, which is close to the previously observed size for full-length Sab protein (Figure 5A, right panel; [10]). A larger species was also recognized in some cell extracts (indicated by an asterisk in Figure 5A), which may correspond to splice variants of Sab [10] or Sab proteins that have undergone post-translational modification(s).

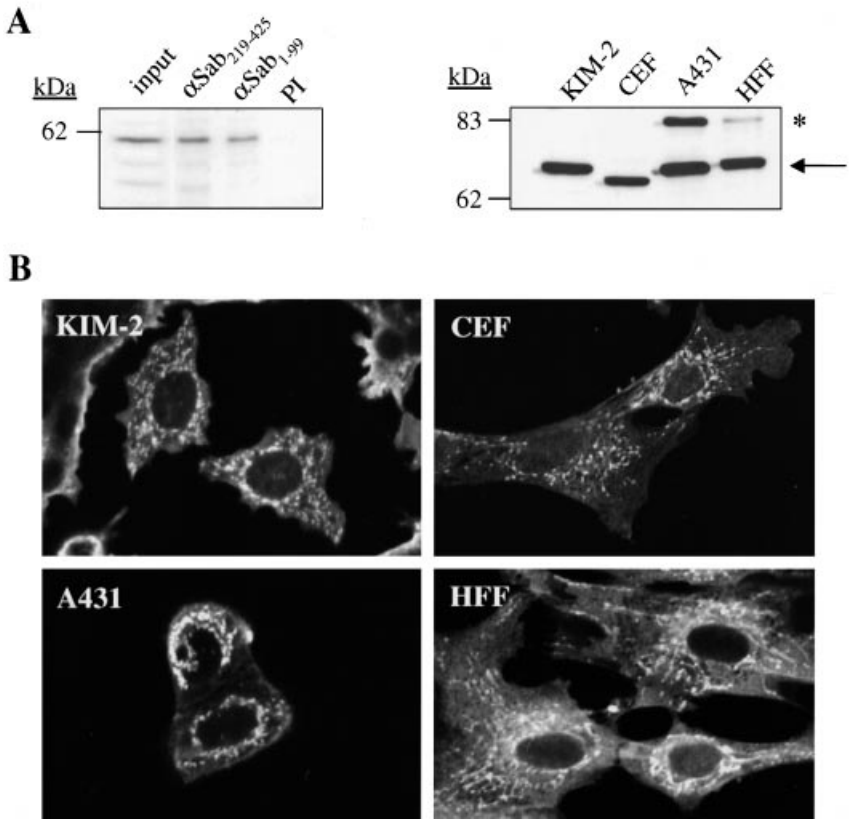

Figure 5 Characterization of Sab protein expression and subcellular localization in human, murine and avian cells

(**A**) Left panel: full-length Sab was synthesized by *in vitro* transcription/translation in the presence of [^{35}S]methionine and immunoprecipitated with anti-Sab-(219–425), the corresponding preimmune serum (PI) or anti-Sab-(1–99). After washing, immunoprecipitated proteins were eluted and analysed by SDS/PAGE followed by autoradiography. Right panel: whole-cell extracts from the indicated cell types were resolved by SDS/PAGE and analysed by Western blotting using the anti-Sab-(219–425) antiserum. The arrow indicates possible full-length Sab proteins, the asterisk indicates possible splice variants of Sab. (**B**) Subcellular localization of Sab in KIM-2 cells, CEF, A431 cells and HFF determined by confocal immunocytochemistry. Cells were grown on coverslips, fixed and immunostained with anti-Sab-(219–425).

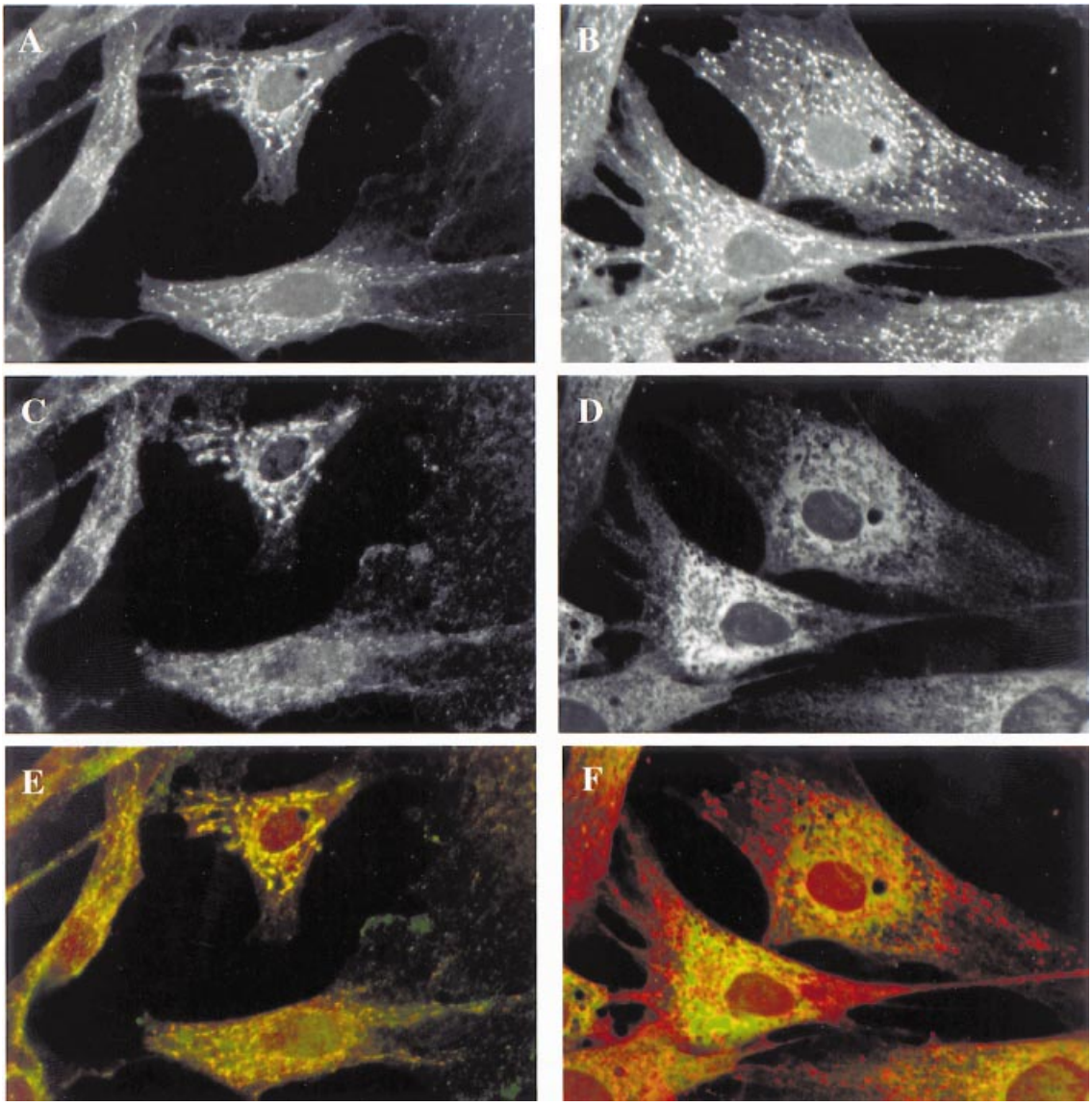

Figure 6 Sab localizes to the mitochondria, not to the endoplasmic reticulum

CEF were dual immunostained with anti-Sab-(219–425) (**A**, **B**) and anti-CoxIV (**C**) or anti-PDI (**D**). Merged images show co-localization of Sab with CoxIV (**E**), but not with PDI (**F**) [red, Sab; green, CoxIV (**C**, **E**) or PDI (**D**, **F**); yellow, co-localization].

No information as to the subcellular localization of Sab has been reported; therefore immunolocalization studies were carried out on the panel of cells analysed by Western blotting. A distinctive pattern of staining was observed in all cell types which indicated that Sab was associated with punctate or strand-like structures that were excluded from the nucleus (Figure 5B). This staining pattern was highly reproducible, and was not observed in cells stained with the preimmune serum or second antibody alone (results not shown).

In order to identify these subcellular structures, co-localization experiments were carried out using antibodies to a number of organelle markers (Figure 6). Antibodies against CoxIV and PDI gave specific and distinct patterns of localization, identifying the mitochondria and endoplasmic reticulum respectively (Figures 6C and 6D). While there was no discernable co-localization of Sab and PDI (Figure 6F), almost complete co-localization was observed between Sab and CoxIV (Figure 6E). That these structures did in fact correspond to mitochondria was confirmed by staining with MitoTracker CMXRos, a dye that accumulates in active mitochondria (results not shown). These results indicate that Sab is associated with mitochondria, a conclusion supported by cell fractionation studies described below.

Sab co-localizes with a subset of active JNK in mitochondria

Since Sab has the potential to bind to active (phospho-)JNK, it was of interest to determine if these molecules associate with the same subcellular structures *in vivo*. In untreated CEF, an anti-phospho-JNK monoclonal antibody exhibited weak cytoplasmic staining, which was punctate and partially coincident with that of Sab (Figures 7A, 7C and 7E). However, unlike Sab, phospho-JNK did not appear to be excluded from the nucleus (Figure 7C).

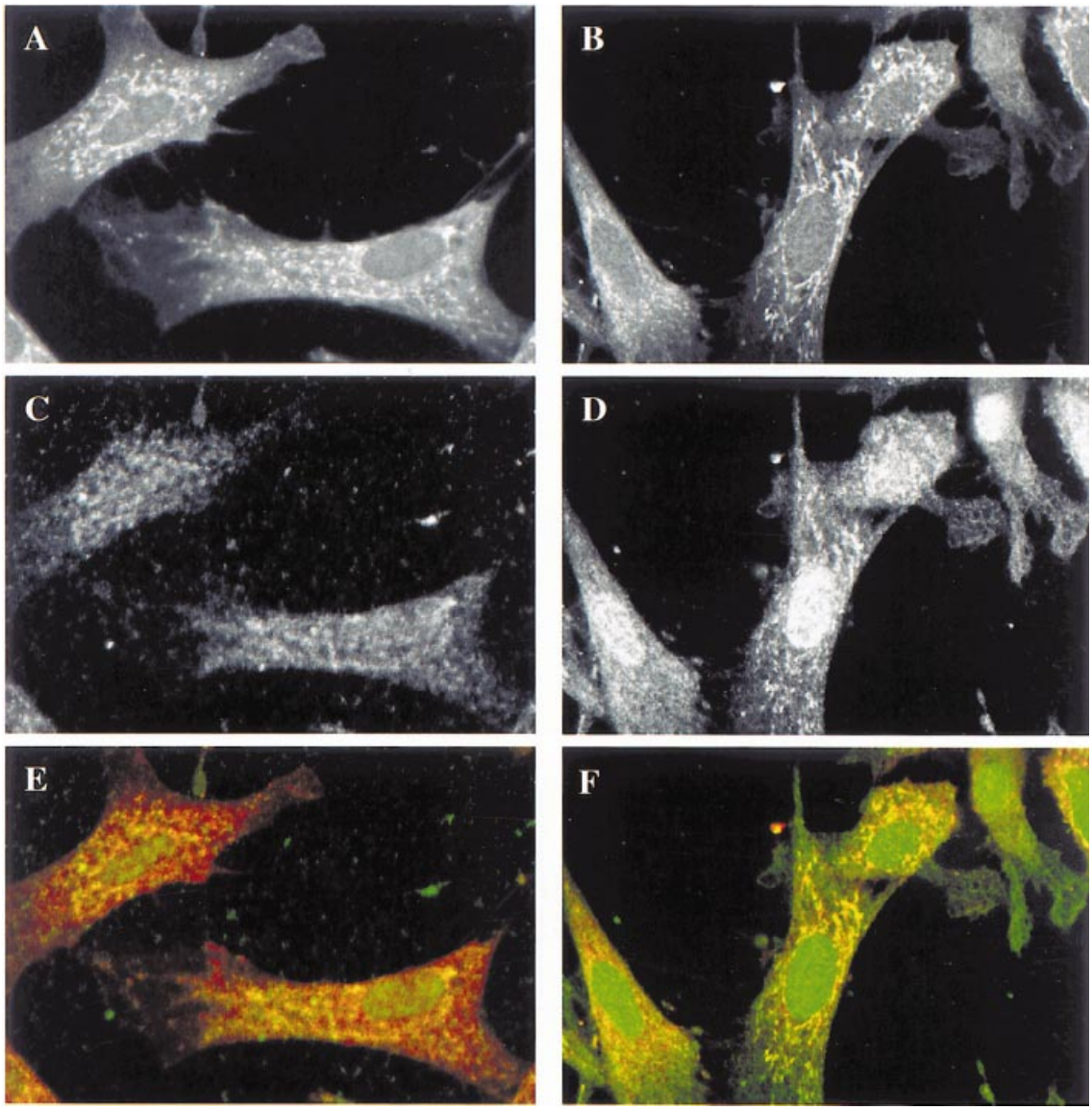

Figure 7 Co-localization of Sab and a portion of phospho-JNK in anisomycin-treated cells

CEF grown on coverslips were fixed either untreated (**A**, **C**, **E**) or after anisomycin treatment (**B**, **D**, **F**) and immunostained with anti-Sab-(219–425) (**A**, **B**) or anti-phospho-JNK (**C**, **D**). Images were merged to assess the extent of co-localization between Sab and phospho-JNK in unactivated (**E**) and activated (**F**) cells (red, Sab; green, phospho-JNK).

When cells were treated with anisomycin to activate JNK, both nuclear and cytoplasmic phospho-JNK staining increased markedly, and in the cytoplasm it was evident that a portion of the active JNK was associated with the same punctate or thread-like structures as Sab (Figures 7B, 7D and 7F). In contrast, no co-localization of Sab with active p38 or ERK MAP kinases was observed (results not shown).

To confirm these observations using an alternative approach, we investigated the distribution of Sab and active JNK by subcellular fractionation. Control and anisomycin-treated CEF were lysed and sequential centrifugation was used to isolate fractions enriched in mitochondria (HM), endoplasmic reticulum plus plasma membranes (LM) and cytosol (Cyto) (Figure 8). The distributions of Sab, active JNK, the mitochondrial marker protein CoxIV and the endoplasmic reticulum marker PDI in these fractions were then determined by Western blotting. This analysis (Figure 8) revealed that Sab was concentrated in the mitochondrial (HM) fraction both before and after stimulation, but that approx. 50% of non-nuclear active JNK was also present in this fraction after anisomycin treatment. No active JNK or Sab was present in the fraction enriched in endoplasmic reticulum (LM). Thus Sab and a fraction of active phospho-JNK co-localize to mitochondria after JNK activation, as judged by both confocal microscopy and biochemical analysis.

DISCUSSION

In the present study we describe the isolation of a new JNK-interacting protein, Sab, which was shown previously to bind selectively to the SH3 domain of Btk [10]. The native Sab protein is 425 amino acids long [10], although our yeast two-hybrid

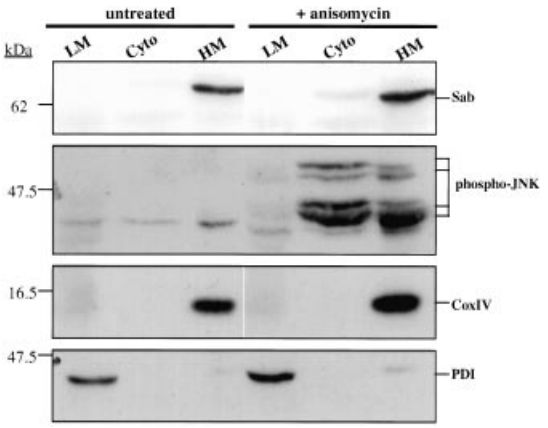

Figure 8 Co-localization of Sab and a portion of phospho-JNK with mitochondria during subcellular fractionation

Growing CEF (left) or CEF that had been treated with anisomycin to activate JNK (right) were fractionated by sequential centrifugation to generate fractions enriched in mitochondria (HM), endoplasmic reticulum plus plasma membranes (LM) and cytosol (Cyto). Equal amounts of protein from each fraction were then resolved by SDS/PAGE and transferred to nitrocellulose, and the presence of Sab, active phospho-JNK, CoxIV and PDI proteins was determined by Western blotting.

screen using JNK3 as a bait retrieved a partial Sab cDNA encoding residues 219–425. This C-terminal segment of Sab exhibits no extended similarity to any other known or predicted protein; however, it contains two short sequences which match the consensus JNK docking (KIM) motif found in c-Jun, and four SP residues that could serve as potential JNK phospho-acceptor sites. As one might predict from these features, Sab can bind to and serve as a substrate for JNK *in vitro*, and has the potential to bind all known JNK isoforms encoded by the *JNK1*, *JNK2* and *JNK3* genes [14].

Interestingly, deletion and point mutants of Sab that lack the most N-terminal of these KIM-like sequences (RPGSLDL) no longer bind to JNK, indicating that this represents a *bona fide* JNK docking motif analogous to that found in c-Jun. In addition, such mutants no longer serve as substrates for JNK, even when they retain all of the four potential SP JNK phosphorylation sites. Thus, as with c-Jun [5], binding of JNK is required for efficient phosphorylation of Sab *in vitro*, although further work will be required to establish whether or not Sab, like c-Jun, serves as a substrate for JNK *in vivo*. Interaction of JNK with Sab could not be detected by co-immunoprecipitation (results not shown). However, this method has also proved unsuccessful for detecting the interaction of JNK with the paradigm (and known physiological) substrate, c-Jun [5], presumably because c-Jun–JNK and Sab–JNK complexes are insufficiently stable to survive the co-immunoprecipitation process, or alternatively, form only transiently *in vivo*. Further work will be required to distinguish between these possibilities; however, it is evident from these results that the JNK binding properties of Sab are comparable with those of c-Jun.

The amino acid sequence of full-length Sab provides few other clues as to its possible function(s). The N-terminal half of the protein exhibits similarity to the rod domain of myosin heavy chain and is predicted to have a high probability of forming an extended coiled coil. Although the significance of this putative coiled-coil region is unknown, full-length Sab can also bind and serve as a substrate for JNK (C. Wiltshire, unpublished work). The minimal region of Sab required for binding to the SH3 domain of Btk is located in the centre of the molecule between amino acids 163–193 [10]. This region lies outside the minimal region required for JNK binding (Figure 1A); however, whether Sab can bind JNK and Btk simultaneously remains to be determined.

The finding that Sab co-localizes with active JNK at mitochondria was unexpected. Association of JNK with mitochondria has been reported previously [26,27], and phosphorylation of Bcl-2 or Bcl-xL proteins has been proposed as a possible mechanism through which JNK signalling could modulate apoptosis via cytochrome *c* release [26,28]. One possibility, therefore, is that Sab might act as an anchor molecule that recruits JNK to mitochondria in order to facilitate phosphorylation of Bcl-2 or other targets. Alternatively, phosphorylation of Sab itself by JNK could affect mitochondrial function in some way. Clearly, it will be important to test these ideas, although such experiments will be complicated by the fact that JNK-mediated phosphorylation has been reported to both inhibit and promote the anti-apoptotic function of Bcl-2 [26,28,29], while JNK activation does not always correlate with Bcl-2 phosphorylation *in vivo*, for unknown reasons [30].

Finally, it is striking that Sab has also been shown to interact with the SH3 domain of the tyrosine kinase Btk [10]. As with JNK, Btk has been implicated in both pro- and anti-apoptotic signalling processes [31–33], although the underlying mechanisms are incompletely understood. Experimental overexpression of Sab inhibits Btk activity [34], suggesting that the Sab–Btk interaction is likely to be physiologically significant. It is well established that Btk can activate the JNK pathway [13], and in certain cases JNK has been implicated as an intermediate in apoptosis triggered by Btk [31]. One possibility, therefore, is that Sab acts as a scaffold that facilitates or co-ordinates cross-talk between the Btk and JNK pathways. Alternatively, Sab might recruit Btk to a specific subcellular location, such as mitochondria, although, unlike with JNK, we are not aware of direct evidence for association of Btk with this organelle. Further work will be required to understand the functional significance of interactions between Sab, JNK and Btk.

We thank R. J. Davis and I. Morgan for providing plasmid constructs and other reagents, J. A. Wyke for comments on the manuscript, and Cancer U.K. and the Association for International Cancer Research for support.

REFERENCES

1 Davis, R. J. (1994) MAPKs: new JNK expands the group. Trends Biochem. Sci. **19**, 470–473
2 Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. Cell **103**, 239–252
3 Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E. and Woodgett, J. R. (1991) Phosphorylation of c-jun mediated by MAP kinases. Nature (London) **353**, 670–674
4 Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M. and Karin, M. (1991) Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. Nature (London) **354**, 494–496
5 Hibi, M., Lin, A., Smeal, T., Minden, A. and Karin, M. (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7**, 2135–2148
6 Sharrocks, A. D., Yang, S. H. and Galanis, A. (2000) Docking domains and substrate-specificity determination for MAP kinases. Trends Biochem. Sci. **25**, 448–453
7 Yasuda, J., Whitmarsh, A. J., Cavanagh, J., Sharma, M. and Davis, R. J. (1999) The JIP group of mitogen-activated protein kinase scaffold proteins. Mol. Cell. Biol. **19**, 7245–7254
8 Kelkar, N., Gupta, S., Dickens, M. and Davis, R. J. (2000) Interaction of a mitogen-activated protein kinase signaling module with the neuronal protein JIP3. Mol. Cell. Biol. **20**, 1030–1043
9 Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A. and Davis, R. J. (2000) Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science **288**, 870–874

10 Matsushita, M., Yamadori, T., Kato, S., Takemoto, Y., Inazawa, J., Baba, Y., Hashimoto, S., Sekine, S., Arai, S., Kunikata, T. et al. (1998) Identification and characterization of a novel SH3-domain binding protein, Sab, which preferentially associates with Bruton's tyrosine kinase (BtK). Biochem. Biophys. Res. Commun. **245**, 337–343
11 Desiderio, S. (1997) Role of Btk in B cell development and signaling. Curr. Opin. Immunol. **9**, 534–540
12 Satterthwaite, A. B., Li, Z. and Witte, O. N. (1998) Btk function in B cell development and response. Semin. Immunol. **10**, 309–316
13 Kawakami, Y., Kitaura, J., Hata, D., Yao, L. and Kawakami, T. (1999) Functions of Bruton's tyrosine kinase in mast and B cells. J. Leukocyte Biol. **65**, 286–290
14 Gupta, S., Barrett, T., Whitmarsh, A. J., Cavanagh, J., Sluss, H. K., Derijard, B. and Davis, R. J. (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. EMBO J. **15**, 2760–2770
15 Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature (London) **369**, 156–160
16 Crouch, D. H., Fisher, F., Clark, W., Jayaraman, P. S., Goding, C. R. and Gillespie, D. A. (1993) Gene-regulatory properties of Myc helix-loop-helix/leucine zipper mutants: Max-dependent DNA binding and transcriptional activation in yeast correlates with transforming capacity. Oncogene **8**, 1849–1855
17 Clark, W. and Gillespie, D. A. (1997) Transformation by v-Jun prevents cell cycle exit and promotes apoptosis in the absence of serum growth factors. Cell Growth Differ. **8**, 371–380
18 May, G. H., Harris, F., Gillespie, D. and Black, D. M. (1999) The BRCA2 transactivation domain does not interact with JNK. Genes Chromosomes Cancer **25**, 407–409
19 Susin, S. A., Larochette, N., Geuskens, M. and Kroemer, G. (2000) Purification of mitochondria for apoptosis assays. Methods Enzymol. **322**, 205–208
20 Sambrook, J., Fritsch, E. F. and Maniatis, T. (eds) (1989) Molecular Cloning: A Laboratory Manual, 2nd edn, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
21 Smith, D. B. and Johnson, K. S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67**, 31–40
22 Clark, W., Black, E. J., MacLaren, A., Kruse, U., LaThangue, N., Vogt, P. K. and Gillespie, D. A. (2000) v-Jun overrides the mitogen dependence of S-phase entry by deregulating retinoblastoma protein phosphorylation and E2F-pocket protein interactions as a consequence of enhanced cyclin E-cdk2 catalytic activity. Mol. Cell. Biol. **20**, 2529–2542
23 Black, E. J., Clark, W. and Gillespie, D. A. (2000) Transient deactivation of ERK signalling is sufficient for stable entry into G0 in primary avian fibroblasts. Curr. Biol. **10**, 1119–1122
24 Kallunki, T., Deng, T., Hibi, M. and Karin, M. (1996) c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell **87**, 929–939
25 Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P. and Flavell, R. A. (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature (London) **389**, 865–870
26 Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., Cheng, K., Chen, Y. N., Campbell, A., Sudha, T. et al. (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. J. Biol. Chem. **275**, 322–327
27 Ito, Y., Mishra, N. C., Yoshida, K., Kharbanda, S., Saxena, S. and Kufe, D. (2001) Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells. Cell Death Differ. **8**, 794–800
28 Yamamoto, K., Ichijo, H. and Korsmeyer, S. J. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. Mol. Cell. Biol. **19**, 8469–8478
29 Ito, T., Deng, X., Carr, B. and May, W. S. (1997) Bcl-2 phosphorylation required for anti-apoptosis function. J. Biol. Chem. **272**, 11671–11673
30 Wang, T. H., Popp, D. M., Wang, H. S., Saitoh, M., Mural, J. G., Henley, D. C., Ichijo, H. and Wimalasena, J. (1999) Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J. Biol. Chem. **274**, 8208–8216
31 Kawakami, Y., Miura, T., Bissonnette, R., Hata, D., Khan, W. N., Kitamura, T., Maeda-Yamamoto, M., Hartman, S. E., Yao, L., Alt, F. W. and Kawakami, T. (1997) Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proc. Natl. Acad. Sci. U.S.A. **94**, 3938–3942
32 Uckun, F. M., Waddick, K. G., Mahajan, S., Jun, X., Takata, M., Bolen, J. and Kurosaki, T. (1996) BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. Science **273**, 1096–1100
33 Vassilev, A., Ozer, Z., Navara, C., Mahajan, S. and Uckun, F. M. (1999) Bruton's tyrosine kinase as an inhibitor of the Fas/CD95 death-inducing signaling complex. J. Biol. Chem. **274**, 1646–1656
34 Yamadori, T., Baba, Y., Matsushita, M., Hashimoto, S., Kurosaki, M., Kurosaki, T., Kishimoto, T. and Tsukada, S. (1999) Bruton's tyrosine kinase activity is negatively regulated by Sab, the Btk-SH3 domain-binding protein. Proc. Natl. Acad. Sci. U.S.A. **96**, 6341–6346

Received 8 April 2002/25 June 2002; accepted 7 August 2002
Published as BJ Immediate Publication 7 August 2002, DOI 10.1042/BJ20020553