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JAMP, a Jun N-Terminal Kinase 1 (JNK1)-Associated Membrane Protein, Regulates Duration of JNK Activity†

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We report the identification and characterization of JAMP (*J*NK1 [Jun N-terminal kinase 1]-*a*ssociated *m*embrane *p*rotein), a predicted seven-transmembrane protein that is localized primarily within the plasma membrane and associates with JNK1 through its C-terminal domain. JAMP association with JNK1 outcompetes JNK1 association with mitogen-activated protein kinase phosphatase 5, resulting in increased and prolonged JNK1 activity following stress. Elevated expression of JAMP following UV or tunicamycin treatment results in sustained JNK activity and a higher level of JNK-dependent apoptosis. Inhibition of JAMP expression by RNA interference reduces the degree and duration of JNK activation and concomitantly the level of stress-induced apoptosis. Through its regulation of JNK1 activity, JAMP emerges as a membrane-anchored regulator of the duration of JNK1 activity in response to diverse stress stimuli.

The stress-activated protein kinases comprise a number of mitogen-activated protein kinase (MAPK) family members that play a central role in regulating cellular responses to various forms of stress (22). These kinases dictate cell fate, i.e., their commitment to apoptosis versus survival in a stress- and cell type-dependent manner (31). Jun N-terminal kinase (JNK) is regulated by a set of upstream MAPK kinases, including the proximal MKK4/7, which activate JNK through phosphorylation on residues 183 and 185 (7, 35). Among the potent activators of JNK are UV irradiation, reactive oxygen species, cytokines, and endoplasmic reticulum (ER) stress (17, 28). ER stress activation of JNK was shown to require the transmembrane protein kinase IRE1 (30) and is similar to that initiated by cell surface receptors in response to extracellular signals (20, 25). JNK has also been implicated in microtubule and cytoskeletal organization, as in the regulation of focal adhesions and cell migration (4, 11, 23).

JNK is among the key players in the cellular stress response which mediates its activities via the phosphorylation of its substrates, c-Jun, ATF2, p53, and Bad, each of which has been implicated in the regulation of cell death and survival (9, 10, 12, 14). The mechanisms underlying JNK's ability to affect cell death depend on its downstream targets acting in concert with different antiapoptosis-signaling pathways, including NF-κB and IAP. For example, JNK-dependent activation of Bid and Smac/DIABLO results in the disruption of the TRAF2-cIAP1 complex, with concomitant attenuation of antiapoptosis signals from cIAP (6). NF-κB was shown to antagonize JNK signaling by targeting and suppressing its upstream kinase, MKK7, with Gadd45b (24). In contrast, JNK can also induce survival signaling by activating JunD, which can collaborate with NF-κB (19). These studies point to the complex interplay among diverse signaling cascades. Furthermore, the immediate proximal regulators of JNK signaling appear to be equally complicated. For example, the degrees and durations of JNK activities are determined by several MAPK phosphatases, including MKP5 (29), as well as by scaffold proteins, including JIP1-4 and POSH (32, 34).

We have identified JAMP (*J*NK1-*a*ssociated *m*embrane *p*rotein) as an RNF5 (RING finger protein 5; an E3 ubiquitin ligase [21] also known as RMA1)-associated protein. Originally identified in *Caenorhabditis elegans* (18), RNF5 binds to and ubiquitinates paxillin, resulting in the exclusion of paxillin from focal adhesions and the inhibition of cell motility (8). Here we identify and characterize JAMP as a novel JNK1-associated protein that affects the duration of JNK activity and affects its ability to induce apoptosis.

MATERIALS AND METHODS

Cell culture and transfection. Adenovirus-transformed human embryonic kidney (HEK) 293T, HeLa, and C2C12 cells and mouse fibroblast NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum or fetal bovine serum (C2C12). All cultures were supplemented with 100 U of penicillin and 100 U of streptomycin (Invitrogen) per milliliter and maintained in a 5% CO_2 incubator at 37°C. Transfection was performed with the calcium phosphate technique for 293T cells and using Lipofectamine Plus (Invitrogen) for HeLa, C2C12, and NIH 3T3 cells according to the respective manufacturers' protocols. Cells were harvested 48 h after transfection. To generate NIH 3T3 cells stably expressing Flag-JAMP, pcDNA-Flag-JAMP was transfected and positive clones were selected with 500 μg/ml of G418 sulfate.

DNA constructs. Full-length mouse *JAMP* cDNA was amplified by PCR and cloned into pcDNA-Flag, pEF-hemagglutinin (HA), pGEX-4T, and pEGFP-green fluorescent protein (GFP) vector. JAMP-N1 and N2 mutants were generated by site-directed mutagenesis on pEGFP-JAMP using primers to N glyco-

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sylation sites at S24A and S136A, respectively. The JAMP C-terminal domain was deleted via PCR-based cloning of the DNA fragment corresponding to amino acids (aa) 1 to 271 of JAMP into pcDNA-Flag. HA-JNK1, JNK2, and Flag-MKP5 were constructed as described previously (3). The integrity of each construct was verified by sequencing.

Yeast two-hybrid screens (10^7 yeast transformants) performed using *Saccharomyces cerevisiae* (MaV203 strain) and RNF5 (cloned in pDBLeu; Invitrogen) as previously described (8) identified JAMP as an RNF5-associated protein.

Cell fractionation. The cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested, and cell pellets were lysed with homogenization buffer (20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml leupeptin, and 10 μg/ml aprotinin) by Dounce homogenization (50 strokes). The homogenate was centrifuged at 200 × *g* for 10 min to remove nuclei and unlysed cells, and the resulting supernatant was centrifuged at 100,000 × *g* for 1 h. Supernatant was regarded as cytosolic fraction. The pellet obtained was solubilized with buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 30 min at 4°C. After extraction, cell lysate was subjected to centrifugation at 10,000 × *g* for 20 min. The supernatant was regarded as the membrane fraction. Total cell lysates were prepared from cells using buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 30 min at 4°C. After extraction, cell lysate was subjected to centrifugation at 10,000 × *g* for 20 min. The supernatant was subjected to further analysis.

Antibodies and immunoprecipitations. Total cell lysates were clarified by centrifugation at 15,000 rpm for 10 min and supernatants incubated overnight at 4°C with anti-HA or -Flag antibodies. Antibodies to HA tag (Covance), Flag tag (Sigma), c-Jun, JNK (Santa Cruz), p-c-Jun, extracellular signal-related kinase (ERK), p38 (Cell Signaling), p-p38 (Sigma), α-tubulin (Sigma), p-ERK (Promega), MKP-5 (Imgenex), and GFP (Molecular Probes) were used according to their manufacturers' recommendations. Immunoprecipitation was performed by incubation for 90 min at 4°C with protein G-agarose (Invitrogen). After washing three times with lysis buffer, proteins were solubilized in 3× Laemmli buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Polyclonal antibodies against JAMP were raised against the bacterial fusion protein glutathione *S*-transferase (GST)–JAMP.

Confocal microscopy. Cells grown on 22-mm^2 coverslips were fixed in freshly prepared 3% paraformaldehyde in PBS for 5 min at room temperature. The cells were then washed three times (5 min each) in PBS, a procedure followed by permeabilization in 0.1% Triton X-100 in PBS (pH 7.4) for 1 min and an additional three 5-min washes in PBS. Cells were then incubated in PBS supplemented with 5% bovine serum albumin for 30 min. The cells were incubated with antibodies (75 μl diluted in PBS containing 0.2% bovine serum albumin, as described in Results) for 1 h at room temperature in a humidity chamber. The cells were washed three times in PBS (5 min each) before incubation with 75-μl drops of Alexa-488- and Alexa-568-conjugated anti-rabbit immunoglobulin G (Molecular Probes) diluted (2 μg/ml) in PBS containing 0.2% bovine serum albumin for 60 min at room temperature in a humidity chamber in the dark. The cells were rinsed three times in PBS, a procedure followed by a rinse in 0.1% Triton X-100 in PBS and three additional rinses in PBS. The coverslips were mounted on glass slides in a Vectashield (Vector Laboratories).

JNK immunokinase assays. HA-JNK1 (0.2 μg) was cotransfected with GFP or JAMP-GFP (0.5 μg each) into 293T cells; 36 h after transfection, cells were treated with 10 μg/ml tunicamycin (TM) for 8 h or with UV (60 J/m^2). Thirty minutes after irradiation, HA-JNK1 was immunopurified with anti-HA antibody. JNK immunokinase assays were carried out using a fusion protein, GST–c-Jun^{1-87}, as a substrate in 1× kinase buffer (20 mM HEPES [pH 7.4], 0.5 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM $NaVO_3$, 5 mM β-glycerolphosphate, 75 mM NaCl, and 0.25% NP-40) as described previously (3). Briefly, 1 μg of GST–c-Jun^{1-87} was incubated with HA-JNK1, which was immunopurified from transfected 293T cells in the presence of 1× kinase buffer containing 1 μCi of [^{32}P]ATP and 25 μM cold ATP for 30 min at 30°C. Phosphorylated GST–c-Jun^{1-87} was separated by SDS-PAGE and transferred to a nitrocellulose membrane, and the amount of phosphorylated GST–c-Jun^{1-87} was detected by autoradiography and quantified with a phosphorimager. The same membrane was used for the immunoblotting of JNK to control for equal immunoprecipitations and for Ponceau S staining of the GST–cJun^{1-87} level to ensure equal amounts of substrate.

Treatment and apoptosis studies. For apoptosis studies, cells were trypsinized and fixed with 70% methanol for 2 h and stained with propidium iodide (50 μg/ml), a procedure followed by analysis of flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson) and the CellQuest program. Apoptosis was assessed by quantifying the percentage of hypodiploid nuclei undergoing DNA fragmentation. Treatment with TM (1 or 10 μg/ml) and UV (60 J/m^2) (time point zero) was followed by the analysis described in Results. The pharmacological inhibitors of stress kinases used were p38 (5 μM; SB 203580), ERK (50 μM; PD 98059), PI3K (50 μM; LY 294002), and JNK (12.5 μM; JNK inhibitor II).

RT-PCR analysis. Total RNA was reverse transcribed using oligo$(dT)_{18}$ primer and a cDNA synthesis kit (Invitrogen). Reverse transcription (RT)-PCR was carried out on cDNA using primers designed to amplify 933 bp of JAMP (forward, 5′ATGGCTGTCGATATTCAACCAGCATGCCTT3′; reverse, 5′AG TCTCGAGTCAGTGTCCATTGGCTCCTTC3′) and 250 bp of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNAs (forward, 5′ACCACAGT CCATGCCATCAC3′; reverse, 5′TCCACCACCCTGTTGCTGTA3′).

Amplifications were carried out for 17 cycles in the presence of [^{32}P]dCTP (30 s each at 92°C and 52°C, and 2 min at 72°C), followed by separation by SDS-PAGE and autoradiography.

Generation of pRS-JAMP. A pair of oligonucleotides containing 19 bp of mouse JAMP (nucleotides 107 to 125 [GAGGACAGAGGACCAATGC]) was generated. The oligonucleotides were annealed and ligated into BglII and HindIII sites of pRETRO-SUPER (pRS) vector. Construct integrity was confirmed by direct sequencing of the plasmid. In all, 60 to 70% of confluent 293T cells were cotransfected with 6 μg of Gag-Pol (pMD.OGP), 5 μg of vesicular stomatitis virus-G (pMD.G), and 8 μg of pBabe-puro-EGFP, pRS, or pRS-JAMP by calcium phosphate precipitation overnight. The viral supernatant was collected after 48 h and filtered (0.45 μm), followed by its immediate use for infection (in the presence of 4 μg/ml of Polybrene). pBabe-puro-EGFP was used to monitor the efficiencies of transfection of 293T cells and infection of NIH 3T3 cells, which were around 75 to 90%.

In vitro binding assay. Bacterially expressed and purified GST, GST-JNK1, or GST-JAMP bound to glutathione beads were first incubated with 1% bovine serum albumin in PBS (1 h at room temperature) followed by incubation with either ^{35}S-labeled JAMP, MKP5, or PP2A, which were in vitro translated using a TNT-coupled reticulocyte lysate system (Promega). Bead-bound material was subjected to four washes (PBS containing 0.25% NP-40, 0.1% β-mercaptoethanol, 2 mM EDTA, and 300 mM NaCl) before being eluted and analyzed by SDS-PAGE followed by autoradiography. The in vitro association between JNK and JAMP, which were used as bacterially expressed proteins, was carried out using His-JNK (25 to 50 ng) and GST-JAMP (1 μg) incubated in vitro for 1 h following washings of glutathione bead-bound material and analysis on SDS-PAGE gels stained with Ponceau, followed by immunoblotting with antibodies to His.

Computer-based analysis of JAMP domains. The TMHMM program (www.cbs.dtu.dk/services/TMHMM) was used for structural analysis (16).

Protein structure accession numbers. The Protein Data Bank accession numbers for JAMP are Q8BI36 and BC002238.

RESULTS

Identification of JAMP as an RNF5-associated protein. On the basis of its association with and effect on JNK, we have designated the new protein JAMP (see data below). JAMP was identified by yeast two-hybrid screening, which was carried out to explore RNF5, the RING finger protein E3 ligase which regulates the localization of cytoskeletal proteins. The association of RNF5 with JAMP was confirmed, and the nature of this interaction is currently being investigated. JAMP consists of seven predicted transmembrane domains, a zinc finger domain, and a putative N glycosylation site (Fig. 1a). Computer-based analysis of JAMP domains using the TMHMM program (16) predicted that the C terminal of JAMP is likely to be within the cytoplasm and expected to serve as the docking site for bound proteins.

Analysis of JAMP expression at the RNA level revealed that it is expressed in a wide range of tissue types, including brain, spleen, thymus, liver, kidney, and testis (Fig. 1b). Elevated expression of JAMP has been found in medulloblastomas (1).

JAMP is glycosylated on the NH_2-terminal residue. The finding of conserved glycosylation sites on JAMP led us to determine whether the protein is indeed glycosylated and the

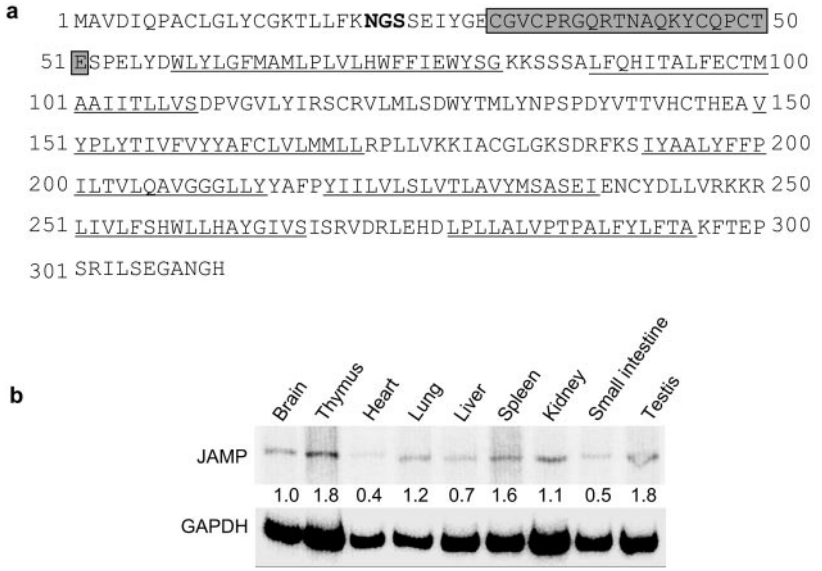

FIG. 1. Structure and expression of JAMP. (a) Amino acid sequence of mouse JAMP. The predicted transmembrane regions are underlined. The N glycosylation site is indicated in bold. The zinc finger domain is shaded. (b) Expression of JAMP in mouse tissues. Total RNA was purified from mouse tissues and reverse transcribed to cDNA, which was used for RT-PCR analysis with 5′ and 3′ primers of JAMP and GAPDH.

significance of this modification. Immunoblot analysis identified two forms of JAMP with the predicted molecular mass (35 kDa), with the higher molecular mass being more abundant (Fig. 2a). TM or thapsigargin treatments (for 18 h) reduced the expression of the form with the higher molecular mass, while increasing the abundance of the species of JAMP with the lower molecular mass. In contrast, UV irradiation did not affect the level of the higher-molecular-mass form of JAMP within 30 min (Fig. 2a), yet it caused an increase in the levels of JAMP at later time points (see below). Since TM as well as thapsigargin is known for its ability to induce ER stress and inhibit protein glycosylation, we further explored the possibility that JAMP may be subject to glycosylation. Treatment with endoglycosidase H also reduced expression of the high-molecular-mass form while elevating the intensity of the lower-molecular-mass form of JAMP (Fig. 2b). These data suggested that the higher-molecular-mass form of JAMP represents the glycosylated form of the protein. Mutation in each of the two putative N glycosylation sites identified the amino-terminal site (N1 mutated on aa 22) to be the N-glycosylated residue (Fig. 2c). These data establish that JAMP is subjected to N glycosylation, which is inhibited after TM treatment and which takes place at the NH_2-terminal site.

Subcellular localization of JAMP. To determine the subcellular localization of JAMP, we monitored the expression of Flag-JAMP via immunoblot analysis of cytosol and membrane fractions prepared using the S100/P100 fractionation protocol (see Materials and Methods). This analysis revealed that JAMP is found primarily within the membrane fraction (Fig. 2d, lanes 2 and 4).

To further assess JAMP localization, we fractionated proteins following treatment with TM or the proteasome inhibitor MG132. The addition of MG132 revealed the presence of nonglycosylated JAMP within the cytosol and membrane fractions (Fig. 2e, lanes 5 and 8), suggesting that cytosolic nonglycosylated JAMP is actively degraded by the proteasome. This result also suggests that localization within the membrane fraction is not dependent on glycosylation. Based on the comparison between the wild-type and N1 forms, it appears that the glycosylation of JAMP primarily affects its stability.

UV treatment increased the amounts of JAMP within the membrane and cytosol fractions (see Fig. S1 in the supplemental material). Such an increase was noted as early as 1 h after UV treatment and was sustained for over 12 h (Fig. 2f). Treatment with TM, however, initially caused an accumulation of JAMP (1 to 3 h) followed by a decrease in the glycosylated form of JAMP (Fig. 2f). These data suggest that the relative expression and the form of JAMP depend on the types of stress stimuli.

To further assess the subcellular localization of JAMP, we performed a series of confocal microscopy-based immunohistochemistry analyses. Antibodies to Flag identified JAMP within the plasma membrane and cytoplasmic organelles of HeLa cells (Fig. 3a). To further pinpoint JAMP localization, Flag-JAMP was coexpressed with GFP-tagged sequences that destine proteins to lysosomes, mitochondria, or ER. As shown in Fig. 3b, Flag-JAMP was found to be localized primarily within the plasma membrane of C2C12 cells that were chosen based on their convenient morphology, which includes a large cytoplasmic compartment. Of interest is the fact that a similar pattern of expression was previously reported for the RNF5 protein, which was used as a bait in the yeast two-hybrid screen, leading to the identification of JAMP (8). In addition to its plasma membrane localization, JAMP was also found to partially colocalize within ER, lysosomes, and the cytoplasm (Fig. 3b). Conversely, JAMP was not found within the mitochondria (see Fig. S2 in the supplemental material). That traces of JAMP could be also found within the cytoplasmic fraction may be attributed to the overexpression of the exogenous protein. The current analysis is also more sensitive than

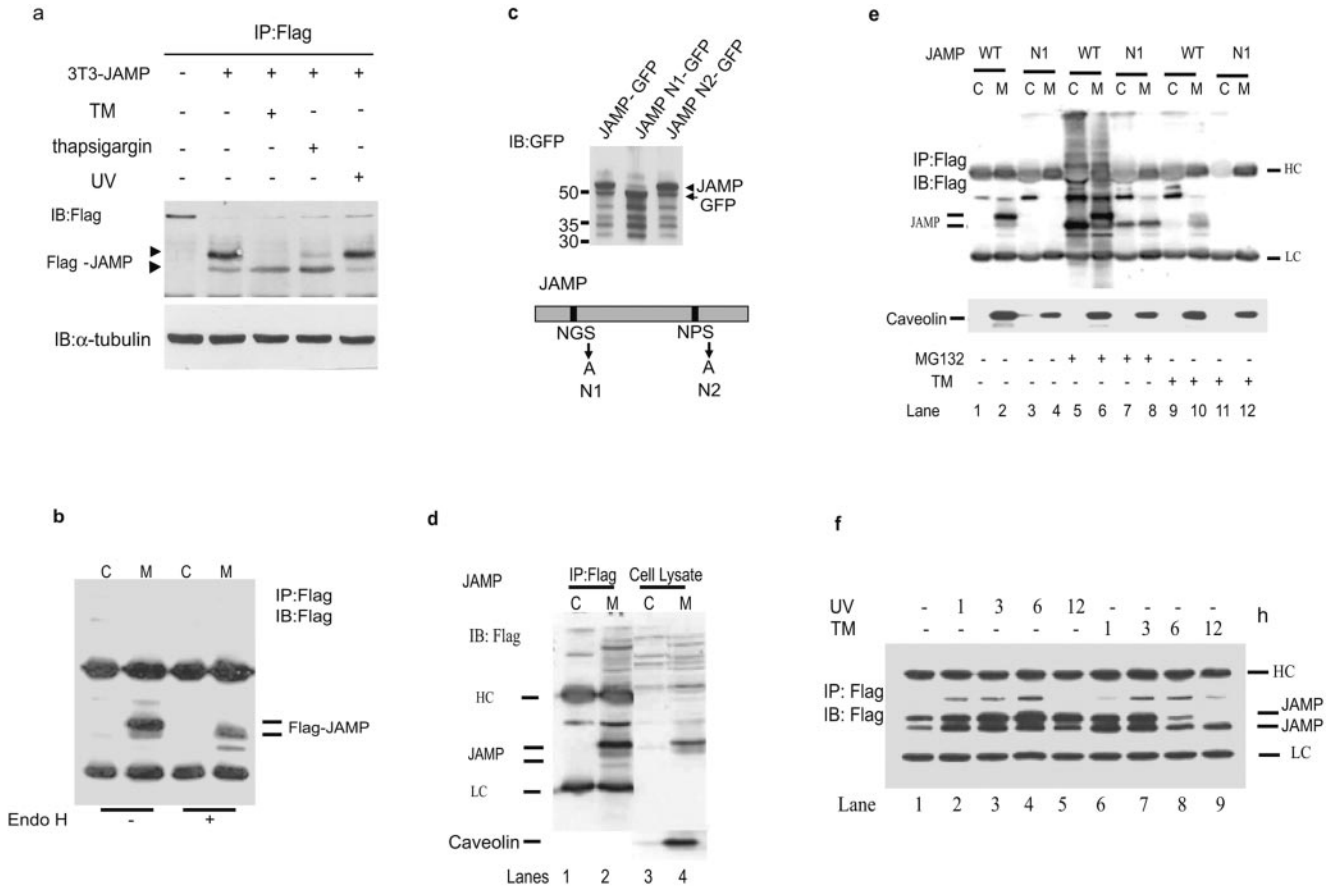

FIG. 2. JAMP is N glycosylated and is primarily localized within the insoluble S100 membrane fraction. (a) JAMP exhibits two major forms which are altered in response to stress. 3T3-JAMP or 3T3 control cells were treated for 16 h with TM (1 μg/ml) or thapsigargin (0.25 μM). Proteins were immunoprecipitated (IP) and immunoblotted (IB) with anti-Flag antibody. (b) Deglycosylation enzyme alters the pattern of JAMP expression. 3T3-JAMP cells were fractionated into cytosolic and membrane proteins which were than used for immunoprecipitation of JAMP using antibodies to Flag. Immunoprecipitates were subjected to Endo-H treatment (5 min) followed by immunoblot analysis with anti-Flag antibody. (c) JAMP is N glycosylated on an NH_2 site. Two putative N-glycosylation sites were mutated as JAMP N1 (aa 22)- or JAMP N2 (aa 134)-GFP, respectively. These mutants were transfected into 293T cells, and their expression patterns were monitored by immunoblot analysis. (d) JAMP localization within the S100 fraction. 3T3-JAMP cells were grown and proteins were prepared as cytosolic (C) and membrane (M) fractions (see Materials and Methods). Proteins (1 mg) were subjected to IP (lanes 1 and 2) followed by IB with anti-Flag antibody. Lanes 3 and 4 represent whole-cell lysates of the indicated cell types. The lower panel depicts the same membrane stripped and reprobed with anti-caveolin antibody as a control to verify the purity of the membrane fraction. (e) JAMP is actively degraded in the cytoplasm. 3T3-JAMP and 3T3-N1 cells were either untreated (lanes 1 to 4)or treated with MG132 (lanes 5 to 8) or TM (lanes 9 to 12). C and M fractions were prepared and subjected to IP followed by IB with antibodies to Flag. The lower panel depicts whole-cell lysates which were subjected to IB analysis using antibodies to caveolin as control for the purity of the S100 fractions. WT, wild type. (f) UV and TM treatment stabilizes JAMP. 3T3-JAMP cells were either untreated (lane 1) or treated with UV or TM for the indicated time intervals before proteins were prepared and subjected to IP with anti-Flag antibody followed by IB with anti-Flag antibody.

Western blots, which did not identify JAMP in the cytosol unless cells were treated with proteasome inhibitor or UV (see Fig. S1 in the supplemental material).

To further assist in following JAMP localization as well as function, we established an NIH 3T3 cell line that stably expresses Flag-JAMP (3T3-JAMP). The level of JAMP expression in these cells was set to be particularly low on the basis of the amount of plasmid transfected, in order to minimize the changes associated with overexpression. Indeed, immunohistochemical analysis of 3T3-JAMP cells performed with Flag antibodies did not identify JAMP expression unless cells were subjected to treatment with proteasome inhibitors, supporting the low level of JAMP expression and suggesting that JAMP is subjected to active proteasome-mediated degradation (see Fig. S3 in the supplemental material).

JAMP associates with JNK1. Changes seen in JAMP expression following TM or UV treatment, both potent inducers of JNK signaling, led us to explore the possibility that JAMP may also affect JNK activity. Since most of the JNK effectors associate with the kinase, we first assessed whether JAMP associates with JNK. Immunoprecipitation of JAMP identified JNK1 but not JNK2 as a JAMP-associated protein (Fig. 4a). Reciprocal immunoprecipitation of JNK1 confirmed its association with JAMP (Fig. 4b). The glycosylation status of JAMP did not appear to affect its association, implying that TM treatment would not affect the ability of JAMP to affect JNK. Deletion of

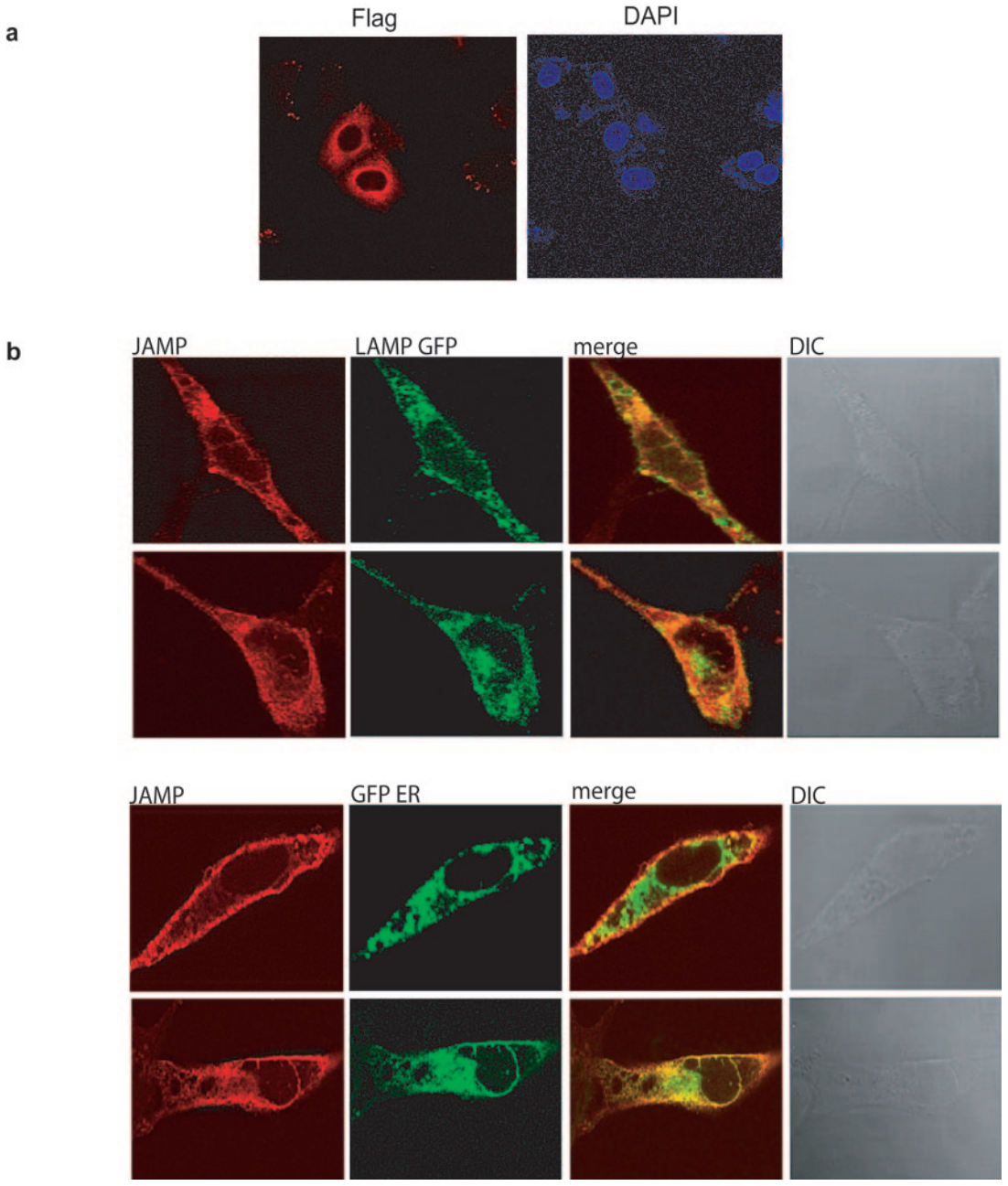

FIG. 3. Subcellular localization of JAMP. (a) Detection of Flag-JAMP distribution. Flag-JAMP was transfected into HeLa cells, and 48 h after transfection, cells were analyzed by means of confocal microscopy-based immunohistochemistry with anti-Flag and anti-JAMP antibodies. DAPI, 4′,6′-diamidino-2-phenylindole. (b) Colocalization of Flag-JAMP with cellular organelles. ER- or lysosome-localizing peptide-GFP was transfected into C2C12 cells, and 48 h after transfection, cells were analyzed by means of confocal microscopy-based immunohistochemistry with anti-Flag (to detect Flag-JAMP) and GFP fluorescence. Also shown are the merged figures and the phase-contrast pictures. LAMP, lysosome-associated membrane protein; DIC, differential interference contrast.

the C-terminal domain of JAMP abolished its ability to associate with JNK (Fig. 4b). This result is consistent with results from computer prediction programs, which suggest that the C-terminal domain is expected to be localized in the outer-membrane cytoplasmic portion where JNK is expected to associate with JAMP. An in vitro binding assay using bacterially produced GST-JAMP and His-JNK1 also confirmed their specific association (Fig. 4c). Analysis of endogenous proteins also confirmed the association of JAMP with JNK. Immunoprecipitation of endogenously expressed JAMP identified JNK prior to as well as after exposure to stress. Interestingly, after exposure to stress in the form of UV irradiation, JAMP was found to associate with the phosphorylated form of JNK (Fig. 4d). Expression of exogenous proteins also confirmed the association of JAMP with JNK prior to as well as after exposure to UV irradiation or TM (see Fig. S4 in the supplemental mate-

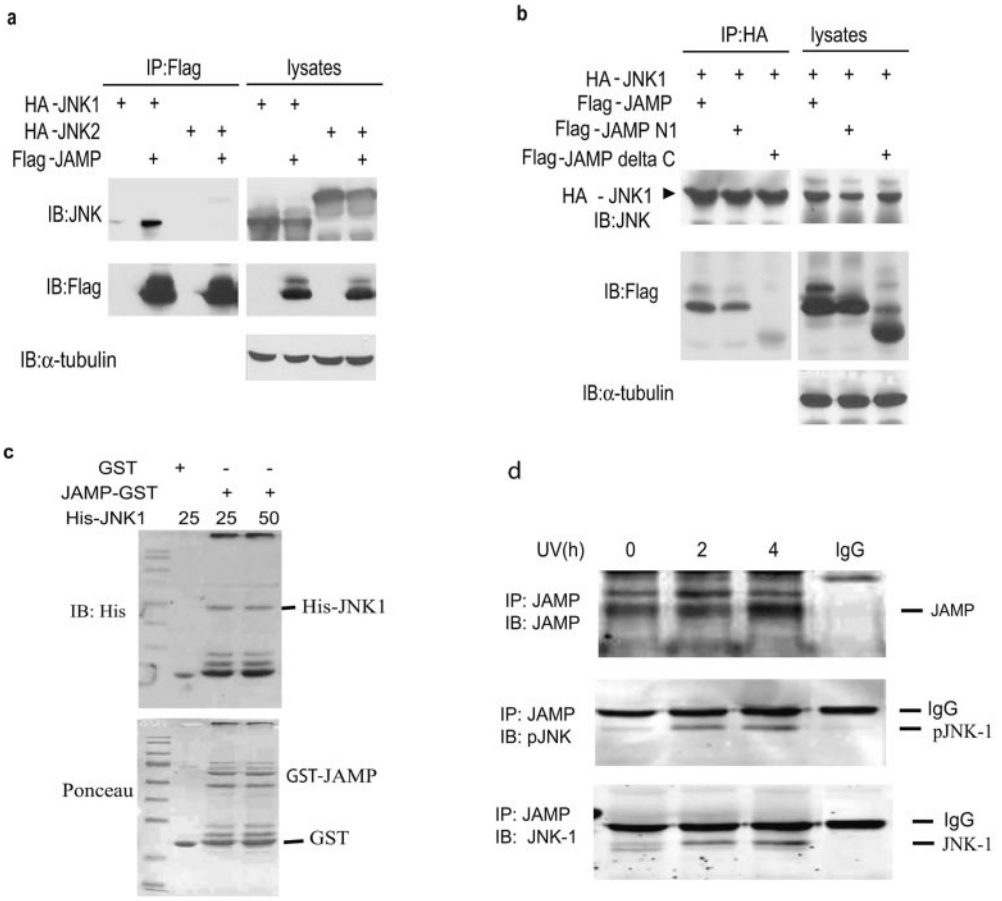

FIG. 4. JAMP interacts with JNK and affects its activity. (a) JNK is a JAMP-associated protein. HA-JNK1, HA-JNK2, and Flag-JAMP were cotransfected into 293T cells, and 48 h after transfection, proteins were prepared and subjected to immunoprecipitation (IP) followed by immunoblot analysis (IB) with the antibodies indicated in the figure. (b) JAMP is a JNK-associated protein. HA-JNK1 and Flag-JAMP, -JAMP N1, or -JAMP delta C (aa 1 to 271) mutants were cotransfected into 293T cells, and 48 h after transfection, proteins were prepared and subjected to IP followed by IB with the antibodies indicated in the figure. (c) JAMP binds to JNK in vitro. Nickel bead-bound His-tagged JNK1 (portions of 25 or 50 ng, as indicated directly above the panel) was incubated with GST-JAMP (or GST as control) for 1 h, followed by washes and analysis of bead-bound material with antibodies to His (upper panel) and Ponceau staining (lower panel). (d) JAMP binds to phosphorylated JNK after UV irradiation. Proteins prepared from 3T3 cells prior to or after UV irradiation were subjected to IP with antibodies to JAMP followed by IB with antibodies to JAMP, JNK, or pJNK.

rial). These data suggest that the association of JNK1 with JAMP is not affected by JAMP modification but rather by the availability of JAMP, which increases following stress.

JAMP affects the degree and the duration of JNK activity. In light of JAMP association with JNK, we have next assessed its possible effect on JNK activity. To this end, we performed immunokinase reactions in which JNK was immunopurified from JAMP-expressing cells. Coexpression of HA-JNK-1 with either Flag-JAMP or JAMP-GFP was sufficient to cause JNK activation, as reflected in the corresponding phosphorylation of GST-c-Jun in vitro (Fig. 5a). These data provide direct evidence for the ability of JAMP to induce JNK activation.

We next assessed changes in basal and inducible JNK activity in 3T3-JAMP cells. The basal level of JNK activity is increased in these cells and further induced following exposure to UV irradiation or serum stimulation. Neither ERK nor p38 was affected upon the expression of JAMP (Fig. 5b), indicating that the JAMP effect is specific for JNK signaling.

To better understand the role of JAMP in the regulation of JNK activity, we have monitored changes in JNK activation at different time points after UV or TM treatment. While JAMP expression caused an additional increase (of approximately twofold) in the level of JNK activity, it had a pronounced effect on the duration of JNK activity. Levels of active JNK remained high 12 h after exposure to UV irradiation in JAMP-expressing cells, whereas in control-transfected cells, JNK activity was reduced to basal levels at this time point (Fig. 5c; also see Fig. S5 in the supplemental material). These findings suggest that JAMP expression potentiates the degree—and prolongs the duration—of JNK activity following UV irradiation.

A more pronounced effect was seen in the case of TM treatment. TM is capable of activating JNK in a cell-type-dependent manner (30). In 3T3 cells, TM causes limited activation of JNK, but only after 24 h (Fig. 5d; also see Fig. S6 in the supplemental material). However, JAMP-expressing cells exhibited sustained JNK activation even after 24 h (Fig. 5d; also see Fig. S6 in the supplemental material). Given that TM

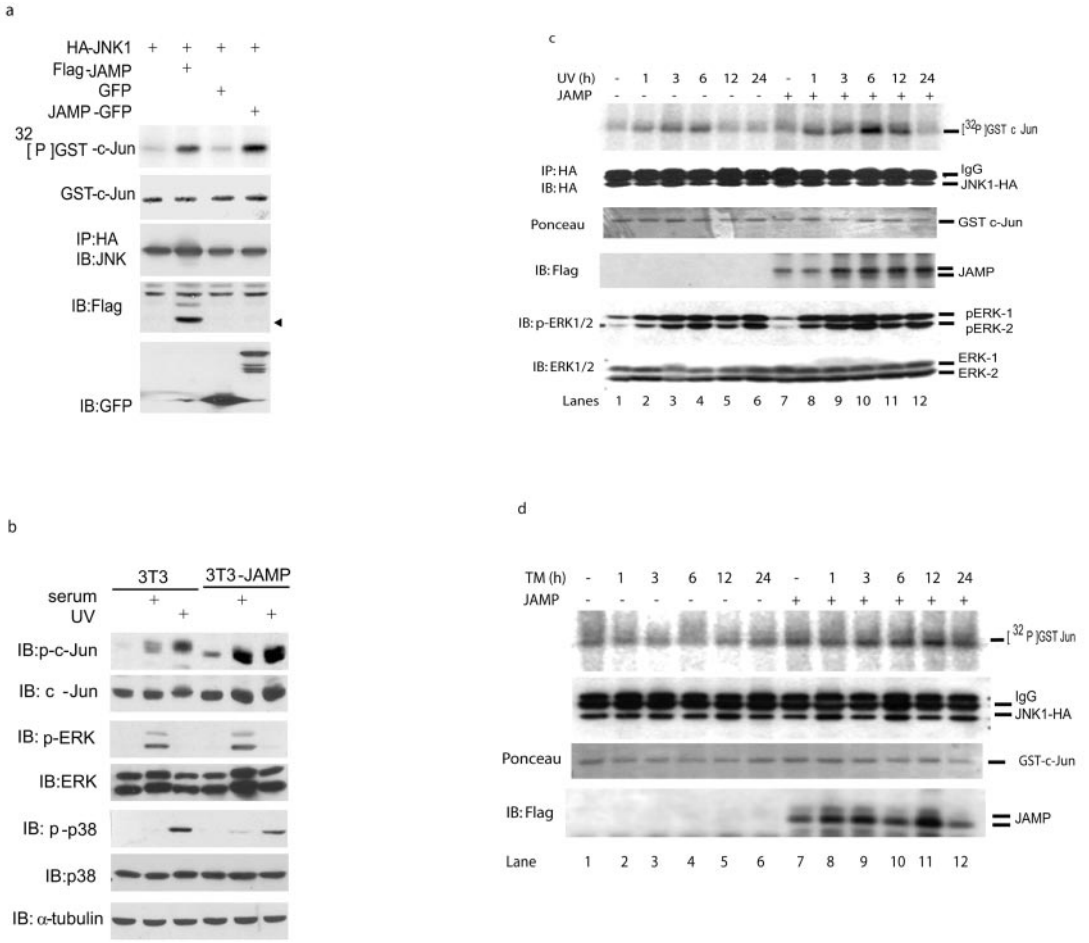

FIG. 5. JAMP affects the degree and the duration of JNK activity. (a) JNK in vitro immunokinase assays. HA-JNK1 was cotransfected with GFP, Flag-JAMP, or JAMP-GFP into 293T cells, and proteins were prepared 48 h later. JNK1 immunoprecipitated (IP) from the cells was used as the kinase for the in vitro phosphorylation of GST-Jun$^{1-87}$. Lower panels depict control Western blots for GST-Jun, which was used as a substrate; the amount of JNK that was immunoprecipitated (IP:HA, IB:JNK) and expression levels of JAMP (Flag and GFP panels). IB, immunoblot analysis. (b) JAMP affects the degree of JNK but not of ERK or p38 activity. 3T3 cells were transfected with HA-JNK1 and Flag-pcDNA or with Flag-JAMP, and 48 h later, cells were treated with UV irradiation or subjected to serum stimulation. Analysis of JNK, ERK, and p38 phosphorylation or activity was performed at the indicated time points using the corresponding phosphoantibodies. Immunoblots with the nonphosphoantibodies were used as control. Antibodies to α-tubulin used to reveal equal loading. (c) JAMP prolongs the duration of JNK activity. 3T3 cells were transfected with HA-JNK1 and Flag- pcDNA or with Flag-JAMP. Cells were treated with UV (60 J/m^2) and were harvested at the indicated time points after treatment. JNK1 immunokinase reactions were carried out using soluble GST-Jun$^{1-87}$ as the substrate. Lower panels depict control Western blot assays for amounts of JNK1, GST-Jun, and JAMP as well as for the effect of JAMP expression on ERK activity. IgG, immunoglobulin G. (d) JAMP induces and prolongs TM-induced JNK activity. The experiment was carried out as indicated for panel a, except that cells were treated with TM (10 μg/ml) for the time intervals indicated.

treatment causes the conversion of JAMP to the nonglycosylated form, it is likely that this form has a more potent effect on JNK.

Activation of JNK1 by JAMP is mediated through competition with MKP5. Since the low expression level of JAMP was found to increase JNK activity even under nonstressful conditions, we assessed possible mechanisms that underlie the effect of JAMP on JNK activity. Among the regulators of JNK is MKP5, a MAPK phosphatase, which selectively affects this signaling cascade (29). We therefore tested the possible effect of JAMP on JNK1's association with MKP5. Significantly, an increased expression of JAMP caused a corresponding decrease in MKP5's association with JNK in vivo (Fig. 6a). Further analysis was carried out in vitro using GST-JNK1, which was incubated with in vitro-translated MKP5 in the presence of increasing amounts of in vitro-translated JAMP. As shown in Fig. 6b, the binding of MKP5 to JNK decreased proportionally to the elevation in the amounts of JAMP that were added to the reaction volume. These data suggest that JAMP decreases the association of MKP5 with JNK1, which may therefore result in an attenuation of MKP5's effect on JNK1, resulting in turn in an increased degree as well as duration of JNK1 activity. The latter data also point to the association of JAMP with JNK in vitro (Fig. 6b). Given the ability of JAMP to outcompete the association of MKP5 with JNK, we have monitored whether the inhibition of JAMP expression by small interfering RNA (siRNA) would affect MKP5's association with JNK in cells subjected to UV irradiation. While MKP5's association

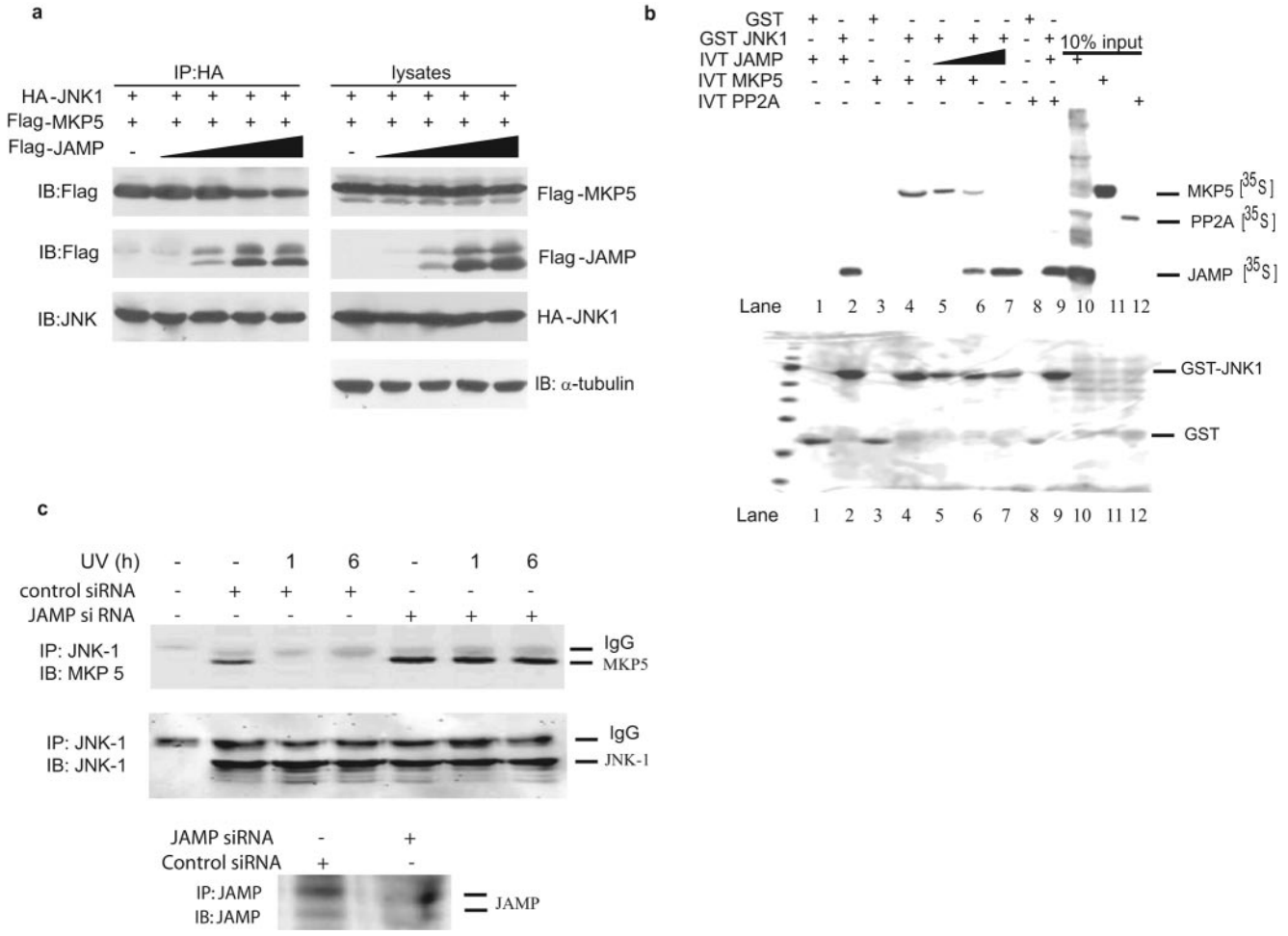

FIG. 6. JAMP affects JNK activity by outcompeting its association with MKP5. (a) JAMP outcompetes MKP5 binding to JNK in vivo. HA-JNK1, Flag-MKP5, and Flag-JAMP were cotransfected into 293T cells. Proteins were prepared 48 h after transfection and subjected to immunoprecipitation (IP) followed by immunoblot analysis (IB) with the indicated antibodies. (b) In vitro analysis of JAMP effect on MKP5-JNK1 interaction. In vitro association was carried out using bacterially expressed and purified GST-JNK1 and in vitro-translated (IVT) and ^{35}S-labeled JAMP and MKP5. Equal amounts (8 μl) of IVT radiolabeled products were incubated with either GST-JNK1 or GST alone followed by their incubation with glutathione beads (4 h at 4°C). Subsequently, GST-JNK1 was extensively washed and resolved by SDS-PAGE before it was transferred to nitrocellulose membrane. GST-JNK-bound ^{35}S-labeled JAMP and MKP5 were detected using a phosphorimager. To check the effect of JAMP on MKP5 binding to GST-JNK1, increasing amounts of radiolabeled JAMP (4 μl [lane5], 8 μl [lane 6], and 16 μl [lane 7]) were incubated with a fixed amount (8 μl) of IVT and ^{35}S-labeled MKP5 (lanes 5, 6, and 7) along with GST-JNK1. In vitro translated and ^{35}S-labeled PP2A (8 μl) was incubated along with radiolabeled JAMP (8 μl) with GST alone and with GST-JNK1 (lanes 8 and 9, respectively) as controls. Lanes 10, 11, and 12 show 10% inputs of IVT JAMP, MKP5, and PP2A, respectively. The lower panel depicts Ponceau S staining of the same membrane. (c) MKP5 binding to JNK increases in cells that were treated with JAMP siRNA. NIH 3T3 cells were infected with either control or JAMP siRNA and were treated 48 h later with UV irradiation (60 J/m^2) for the indicated intervals of time before harvesting. JNK-1 was immunoprecipitated from the cell lysate, followed by IB with anti-MKP5 antibody (upper panel) and anti-JNK-1 antibody (lower panel). The degree of JAMP inhibition by this siRNA is estimated to be about 50% at both protein and RNA levels (see subsequent figure legends for details).

with JNK decreases after UV irradiation, the inhibition of JAMP expression resulted in the increased association of JNK1 with MKP5 after UV treatment, as shown in Fig. 6c. These data further support the competition between JAMP and MKP5 for association with JNK, which affects the duration of JNK activity.

Inhibition of JAMP expression shortens the duration of JNK activity. Since JAMP induces JNK activity, we next determined changes in JNK activation in cells treated with JAMP siRNA to inhibit endogenous JAMP expression. Selected siRNA constructs caused more than 60% inhibition of JAMP expression (Fig. 7a). Cells whose JAMP expression had been inhibited no longer exhibited high basal levels of JNK activity (Fig. 7b).

Using JAMP siRNA, we next reduced JAMP levels in 3T3 as well as in JAMP-3T3 cells. The inhibition of JAMP expression in JAMP-3T3 cells reduced the duration and the level of UV-induced JNK activity (Fig. 7c; also see Fig. S7 in the supplemental material). The inhibition of endogenous JAMP in 3T3 by siRNA also shortened the duration of JNK activity (from 6 to 3 h [Fig. 7d; also see Fig. S8 in the supplemental material]). The reduced magnitude of changes seen in this reaction could be attributed to the lower levels of JAMP expression (endogenous JAMP compared with exogenously expressed JAMP in

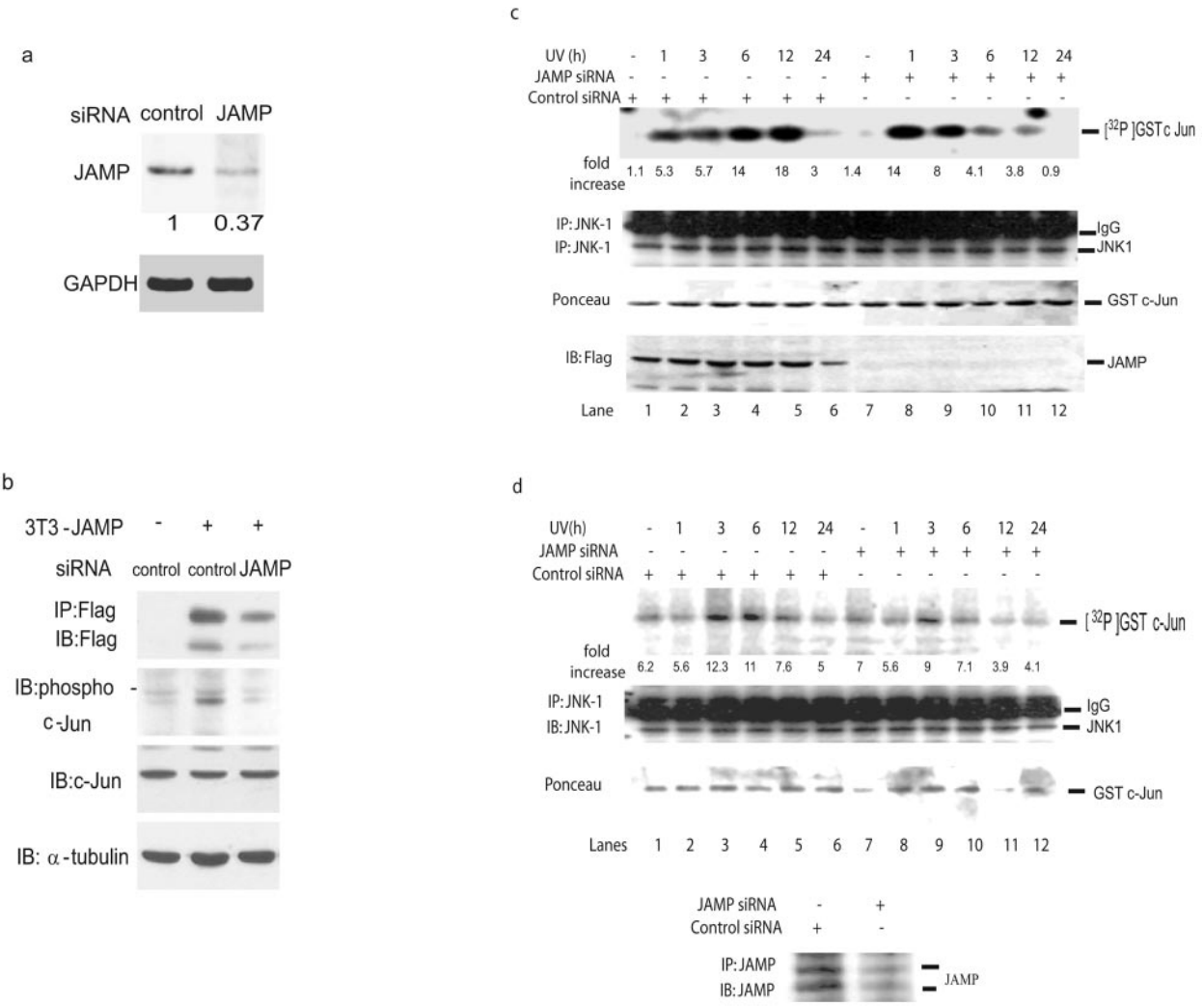

FIG. 7. JAMP is required for stress-induced JNK activation. (a) JAMP siRNA inhibits JAMP expression. NIH 3T3 cells were infected with pRS-JAMP or control (nonrelevant) siRNA, and cells were harvested after 48 h. The prepared RNA was used for RT-PCR analysis to monitor changes at the level of JAMP transcripts. (b) Effect of JAMP on JNK activity. NIH 3T3 cells were infected with JAMP or control (nonrelevant) siRNA. Cells were harvested, and the proteins prepared were subjected to immunoblot analysis (IB) using the indicated antibodies. IP, immunoprecipitation. (c) 3T3-JAMP cells were infected with pRS-JAMP or control (nonrelevant) siRNA. After UV treatment, cells were harvested at the indicated time points. JNK-1 immunokinase reactions were carried out using GST-Jun$^{1-87}$ as a substrate. The lower panel depicts the control Western blots for the expression of JAMP and the amount of JNK that was immunoprecipitated. IgG, immunoglobulin G. (d) JAMP siRNA shortens the duration of endogenous JNK activation by UV. 3T3 cells were infected with pRS-JAMP or control (nonrelevant) siRNA. After UV treatment (at the indicated time intervals), cells were harvested. JNK-1 immunoprecipitated from the cells was used for immunokinase reactions in vitro using GST-Jun$^{1-87}$ as a substrate. Lower panels depict the control Western blots for the amount of JNK that was immunoprecipitated and for GST-Jun, which was used as a substrate. The bottom panel depicts levels of cellular protein extracts, which were analyzed to monitor changes at the level of endogenous JAMP expression upon siRNA for control of JAMP.

JAMP-3T3 cells used for the experiment whose results are shown in Fig. 7c). Similarly, the inhibition of JAMP expression by siRNA reduced the level of TM-induced JNK activity (see Fig. S9 and S10 in the supplemental material). These findings provide direct support for the role of JAMP in regulating the duration of JNK activity.

JAMP is required for stress-induced apoptosis. To assess the biological implications of JAMP activities in the cellular stress response, we treated cells with TM or UV irradiation and monitored changes in cell susceptibility to apoptosis.

Transient expression of JAMP-GFP was sufficient to induce a marked degree (>30%) of apoptosis. The level of apoptosis was similar to that seen upon treatment with TM and yet was elevated (to 57%) upon their combination (Fig. 8a). UV irradiation also resulted in a pronounced apoptosis (>40%), which was further increased upon the combination with JAMP (Fig. 8a). These findings suggest that JAMP expression suffices to induce basal apoptosis and that JAMP also augments the degree of apoptosis induced by TM and UV treatments.

We next assessed changes in apoptosis upon the inhibition of JAMP expression. Cells infected with the control siRNA construct underwent efficient apoptosis following TM or UV treat-

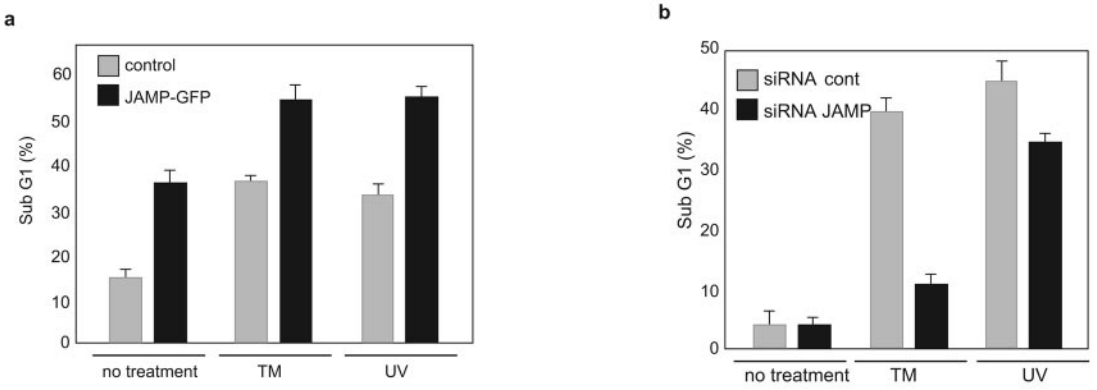

FIG. 8. JAMP is required for stress-induced JNK activation and concomitant apoptosis. (a) Transient expression of JAMP induces apoptosis and augments TM- or UV-induced apoptosis. GFP and JAMP-GFP were transfected into NIH 3T3 cells, and the degrees of apoptosis were monitored. Twenty-four hours after transfection, cells were treated with TM (10 μg/ml) or UV (60 J/m^2), and the degrees of cell death (Sub G1) were monitored 36 h later. (b) JAMP siRNA inhibits TM- and UV-induced apoptosis. NIH 3T3 cells infected with JAMP or control siRNA were treated with 10 μg/ml of TM or 60 J/m^2 of UV for 36 h. Cells were subjected to cell cycle analysis with fluorescence-activated cell sorting. Data are representative of triplicate experiments.

ment (>40%). However, the degree of TM-induced apoptosis was efficiently attenuated in cells whose JAMP expression was inhibited (from 40 to 10%; Fig. 8b). Inhibition of JAMP expression reduced the degree of apoptosis seen following UV treatment, albeit to a lesser degree (from 45% to 35%; Fig. 8b). These data suggest that JAMP is required for the apoptosis induced by UV stress and even more by ER stress.

To assess whether JAMP-induced apoptosis depends on JNK signaling, we have compared the effects of pharmacological inhibitors of JNK, p38, ERK, and PI3K activities, to determine their respective abilities to attenuate such apoptosis. Only the JNK inhibitor could attenuate the degree of JAMP-induced apoptosis (from 35% to 25%; also see Fig. S11 in the supplemental material). Partial inhibition could be due to insufficient inhibition or the effect of JAMP on other signaling pathways, which are possibilities yet to be investigated. Together, these findings provide important support for the role of JAMP in regulating JNK signaling in response to diverse stress stimuli.

DISCUSSION

The present study identifies and characterizes JAMP, a putative seven-transmembrane protein that associates with JNK and affects its activities. JAMP lacks a membrane-targeting signal peptide, yet is localized within the plasma membrane fraction as well as in the ER and lysosomes; JAMP can be also found within the cytoplasm when overexpressed or in its unstable nonglycosylated form. Along these lines, JAMP-associated protein RNF5 (our unpublished data) is also localized within the plasma membrane in mammalian cells (8) and in *C. elegans* (2).

JAMP increases the activity of JNK1 through its ability to squelch JNK phosphatase MKP5. Our data reveals that through the outcompeting of MKP5's association with JNK, JAMP affects the duration of JNK activity. Whereas the overexpression of JAMP prolongs the duration of JNK activation, the inhibition of JAMP by the corresponding siRNA shortens the duration of JNK activity. Changes in JAMP expression are therefore expected to affect the duration of JNK signaling. Indeed, UV irradiation increases the level of JAMP expression, thereby increasing the relative amount of the protein available for association with, and for having an effect on, JNK. While TM also increases JAMP expression within 1 to 3 h, it later decreases the glycosylation of JAMP, resulting in the predominant expression of the nonglycosylated form. While glycosylation does not appear to alter JAMP's effect on JNK, the nonglycosylated form may be more potent in activating JNK, given the changes seen upon TM treatment, which on their own had a marginal effect on JNK activity in the cells used here.

The association of JAMP with and the effect of JAMP on JNK1 also suggest that a specific subset of JNK may be affected. The degree of JNK phosphorylation may also affect its affinity for association with JAMP. Accordingly, JAMP may associate with a subset of JNK isoforms (or posttranslationally modified JNK) which could affect specific substrates, i.e., substrates that are colocalized within insoluble cellular domains where JAMP colocalizes with JNK1. Those are likely to include cytoskeletal proteins, such as paxillin, which was shown to serve as a JNK substrate (15) and as an RNF5 target for ubiquitination (8); both JNK and RNF5 alter paxillin activity. Although further studies are required to delineate substrate differences among the two forms of JNK, tumorigenicity studies have revealed opposing roles for JNK1 and -2 in skin tumorigenesis (5, 27), suggesting that a selective increase in JNK1 activities could coincide with a selective advantage in the development and progression of certain tumor types. Since elevated expression of a spliced form of JAMP lacking glycosylation and zinc finger domains was reported in medulloblastomas (1), this tumor type may serve to identify the nature of differences among JNK substrates. Recent studies pointed to functional differences between JNK1 and JNK2. Whereas JNK2 appears to regulate c-Jun stability, primarily under nonstressful conditions, JNK1's primary function is to elicit the activation of c-Jun after stress (26). Thus, the selective association of JAMP with JNK1 may further explain the mechanism

underlying the functional differences between the two forms of JNK.

The implications of elevated JNK1 activity upon JAMP expression are demonstrated at the level of cell survival both before and after exposure to stress. The elevated expression of JAMP results in increased basal apoptosis that is JNK dependent, suggesting that JAMP provides a proapoptosis signal to cells in which it is transiently expressed. Indeed, a protection against stress-induced apoptosis is seen in cells whose JAMP expression was inhibited by specific siRNA. The latter finding also indicates that the ability of JAMP to increase JNK activity is not likely to be due to stress caused upon JAMP overexpression. Interestingly, TM-induced apoptosis appears to depend on JAMP to a degree greater than that induced by UV. The latter could be explained by the multiple signaling pathways that are induced to induce apoptosis by UV irradiation as opposed to that induced by ER stress, suggesting that JAMP may play a more central role in ER-induced apoptosis.

In light of the effect of JAMP on JNK, several possibilities emerge regarding the physiological and functional significance of this interaction. It is expected that changes in the expression (and glycosylation) of JAMP would determine the degree of its effect on JNK activity. This model stipulates that stress would affect the level of JAMP expression or glycosylation. The model is supported by the observation that UV and TM initially increase the level of JAMP glycosylation, whereas at later time points TM reduces that level.

Although the low level of JAMP expression and the limited specificity of the antibodies against it limit the analysis of endogenous JAMP, glycosylation and other posttranslational modifications could be equally important parameters which would affect the degree/duration of JNK activity. Given the localization of JAMP within the membrane domains, it is possible that the complex of JAMP/JNK is localized within membrane domains that may include TRAF proteins that elicit similar activations of stress kinases and apoptosis. Consistent with this possibility is the observation that TRAF7, a RING-, zinc-, and WD40 domain-containing protein, activates AP1 and induces apoptosis, a profile similar to the effects after JAMP expression (33). Along these lines, TRAF2's ability to activate JNK is associated with its ubiquitination-dependent translocation to the insoluble membrane/cytoskeletal fraction (13), suggesting that JAMP may be part of a greater signaling network. Since interferon gene regulatory element activation of JNK after ER stress was shown to require TRAF2 (30), it is possible that TRAF proteins assemble functional complexes that determine the activation as well as the duration of ER stress-elicited signals. JAMP may be part of such a complex, based on its association with and effects on JNK.

Overall, these studies position JAMP as part of newly recognized layer which contributes to the duration of JNK1 activity and which is likely to affect a select subset of JNK substrates, given the association of JAMP with membrane fractions.

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