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Identification of New JNK Substrate Using ATP Pocket Mutant JNK and a Corresponding ATP Analogue*

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Modification of the ATP pocket on protein kinases allows selective use of an ATP analogue that exhibits high affinity for the altered kinases. Using this approach, we altered the ATP-binding site on JNK and identified N^6-(2-phenythyl)-ATP, a modified form of ATP that exhibits high specificity and affinity for the modified, but not the wild type form, of JNK. Using modified JNK and its ATP analogue enables the detection of novel JNK substrates. Among substrates identified using this approach is heterogeneous nuclear ribonucleoprotein K, which is involved in transcription and post-transcriptional mRNA metabolism. The newly identified substrate can be phosphorylated by JNK on amino acids 216 and 353, which contribute to heterogeneous nuclear ribonucleoprotein K mediated transcriptional activities.

The family of mitogen-activated protein kinases (MAPK)[1] consists of evolutionarily conserved proteins that play a central role in protecting cells from stress and DNA damage (1–5). Major components within the MAPK family are extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPK/JNK), and p38 (5, 6). Whereas ERKs are preferentially activated by mitogens (7, 8), the JNK and p38 pathways are triggered primarily by inflammatory cytokines and by a diverse array of cellular stresses, including UV light and hydrogen peroxide (9–12). Each stress kinase is activated by a defined set of upstream protein kinases that are selectively triggered by signals elicited from cell surface receptors or membrane-anchored proteins, or changes due to altered balance of reactive oxygen species (13, 14). MAPKs phosphorylate proteins located at the plasma membrane, cytoplasm, and nucleus (15). In non-stimulated cells, MAPKs are largely cytoplasmic. Upon activation, a portion of the MAPKs translocates to the nucleus (16–21). The duration of activation of MAPKs influences the extent of their nuclear translocation and, thus, their access to transcription factors (22–27).

Stress-activated protein kinases are encoded by three genes (*JNK 1–3*; reviewed in Ref. 28) that are alternatively spliced to create more than 10 isoforms (29). Whereas *JNK1* and *JNK2* are expressed in most human tissues, *JNK3* is seen primarily in brain, heart, and testis. Originally found as microtubule-associated kinases (30), stress-activated kinases were found to bind the amino-terminal domain of c-Jun (31) and to phosphorylate c-Jun on Ser-63 and -73 (32). In normal growing cells, JNK activity as a kinase is limited by the inhibitory effect of GSTpi (33), and yet it efficiently targets ubiquitination and degradation of its associated proteins, as demonstrated for c-Jun, ATF2, and p53 (34–37). In response to stress, JNK phosphorylation on both Thr-183 and Thr-185 residues by MKK4/7 (9, 27) leads to phosphorylation of JNK substrates, which include c-Jun (38, 39), ATF-2 (40, 41), c-Myc (42), Bcl2 (43), and p53 (36, 44, 45).

For a family of genes that are ubiquitously expressed in more than 10 isoforms, the number of substrates identified to date is surprisingly limited. Attempts to identify JNK substrates by the two-hybrid screen in most cases failed, probably because of the nature of JNK-targeted ubiquitination of its bound substrates. To identify new putative substrates for this kinase, we adopted the approach developed by Shokat and colleagues (46–49), wherein the ATP pocket in a given kinase can be altered so that it exhibits high affinity for selected forms of ATP. This approach enables performing kinase reactions in the presence of whole cellular proteins and yet selectively identifying specific substrates, thus providing a "semi" *in vivo* setting for identifying putative substrates. Here we describe the use of this approach to modify JNK, identify the highest affinity ATP analogue, and subsequently identify putative new substrates. We demonstrate the ability of this technique to identify heterogeneous nuclear ribonucleoprotein K (hnRNP-K, or K protein) as a new MAPK/JNK substrate.

hnRNP-K (K protein) belongs to a large family of nuclear RNA-binding proteins that form complexes with RNA polymerase II transcripts (50, 51). The hnRNP-K protein has been implicated in diverse molecular and cellular functions, including nuclear-cytoplasmic shuttling and RNA transcription and translation (52). The K homology motifs, originally found in hnRNP-K, are implicated in RNA binding (53). Import and export of K protein is mediated via the KNS domain (aa 323–390), which confers bidirectional transport across the nuclear envelope and represents a novel shuttling pathway (54). The K protein has also been shown to regulate translation in the cytoplasm. Together with another K homology domain protein, hnRNP-E1, it binds to a CU-rich "differentiation control element" in the 3′-untranslated region of *15-lipoxygenase* mRNA

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; hnRNP-K, human nuclear ribonucleoprotein K; aa, amino acid(s); SH3, Src homology domain 3; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; wt, wild-type.

and silences the translation of this message in immature erythroid precursor cells (55).

The mammalian (53, 56) and the *Drosophila melanogaster* homologues (57) of K protein have been implicated in transcription. Transactivation by the K protein involves an increase in RNA synthesis of various reporter genes (56). The K protein has also been found to bind to a single-stranded DNA sequence of the human c-*myc* promoter and to affect transcription of c-*myc* as well as of *Sp1* and *Sp3* (58). Interconversion of duplex and single-stranded DNA (59) and association with the C/EBPβ (60) are among the mechanisms that may explain the effect of K protein on transcription.

The mechanistic basis of regulation of K protein activities is not well understood. The carboxyl terminus of the K protein consists of the SH3-binding cluster, which is required for binding of K protein with Vav, an association implicated in the cytoplasmic localization of K protein (61, 62). K protein has been shown to associate with c-Src (63, 64), through which it has been implicated in regulating processing, trafficking, or translation of mRNA. Src as well as protein kinase Cδ and an interleukin 1-responsive K protein kinase have been shown to phosphorylate hnRNP K, although the significance of this phosphorylation is not known (65, 66). Here we characterize the phosphorylation of K protein by JNK and demonstrate that K protein phosphorylation is required for its contribution to AP1-dependent transcriptional activities.

MATERIALS AND METHODS

Cells—Human embryo kidney cells (293T) were maintained in Dulbecco's modified Eagle's medium supplemented with calf serum (10%) and antibiotics.

Plasmids—HnRNP K cDNA was polymerase chain reaction amplified using an HA-tagged primer and cloned into pCDNA3 or pGEX-4T-2. Mutations on serine phosphorylation sites (S116A, S216A, S284A, and S353A) of hnRNP K and in the ATP pocket of JNK2 (M108G and L168A) were performed using the Quick Change Site-directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing. Mutated hnRNP K cDNA was also subcloned into the pGEX-4T-2 plasmid. GST-Jun was expressed and purified as described previously (28). pGEX-4T-2-hnRNP K fusion protein was expressed in *Escherichia coli* BL21 and purified using immobilized glutathione beads (Pierce) by standard methods.

^{32}P Labeling of Phenythyl-ADP—E. coli nucleoside diphosphate kinase (0.1 mg) was mixed with 100 μCi of [γ-^{32}P]ATP (6000 Ci/mmol, Amersham Pharmacia Biotech) in HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) containing 5 mM $MgCl_2$ and equilibrated at room temperature for 5 min followed by separation on a Bio-spin column (p-6). Phenythyl-ADP (5 μl of 0.1 mM) was added to the phosphorylated nucleoside diphosphate kinase for 10 min at room temperature before the reaction mixture was heated (80 °C for 2 min) and subsequently spun down to pellet the denatured nucleoside diphosphate kinase (24).

Protein Kinase Assays—Protein kinase assays were carried out using a fusion protein (GST-Jun) or whole cell extract that was dialyzed against kinase buffer (20 mM HEPES, pH 7.4, 0.5 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM NaF, 0.1 mM $NaVO_3$, 5 mM β-glycerolphosphate, and 75 mM NaCl) as described previously (33). Briefly, 2 μg of GST-Jun or 100 μg of lysate were incubated with immunopurified HA-JNK or HA-JNK-as3 in the presence of kinase buffer containing 3 μCi of [γ-^{32}P]ATP or ^{32}P-labeled N^6-(2-phenythyl)-ATP and 25 μM cold ATP for 30 min at 30 °C. Phosphorylated GST-Jun was separated on SDS-PAGE, and phosphorylated lysate was separated by two-dimensional PAGE followed by staining and autoradiography.

Two-dimensional Gel Electrophoresis—Isoelectric focusing (IEF) gels (consist of 8 M urea, 4% acrylamide/bisacrylamide, 2% Nonidet P-40, 1.6% Pharmalyte pH 5–8, and 0.4% Pharmalyte pH3–10) were prepared in gel tube (inner diameter: 2 mm × 18 cm; BioRad). Following polymerization (3 h), IEF gels were pre-run for 1 h at 200 V with 10 mM H_3P0_4 as anode butter and 20 mM NaOH as cathode buffer. Samples were loaded (40 μl) and run at 400 V for 14–16 h followed by 800 V for 1 h. IEF gels were removed from tube and equilibrated in SDS sample buffer for 15 min at room temperature before they were placed on 12% SDS-polyacrylamide gel and run at 40 mA until the dye front reached to the bottom of the gel.

FIG. 1. **Rationale for mutating specific residues within ATP pocket of JNK.** Former modifications of both tyrosine and serine/threonine kinases from a variety of protein kinase families including, *Src, Fyn, CDK2, Cla4* (yeast Pak kinase), *CaMKII,* and *fus3* (yeast mapk), led to the identification of two key residues that must be modified in wild-type kinase to allow recognition of ATP analogs modified at the N-6 position. These two residues in JNK are M108G and L168A, respectively, and are analogous to the mutations required to create Cla4-as3.

Microsequencing—Tandem nanoflow electrospray mass spectrometry employing a PE Sciex QSTAR instrument was used to determine the sequence of peptides obtained by tryptic digestion of 3 silver-stained spots. The peptide sequences obtained for the corresponding spots were: GGRGGSRAR, NTDEMVELR, and NLPPPPPPR, each of which exhibited 100% identity with the hnRNP-K protein.

Purification of JNK for Kinase Assay—293T cells were transfected with pcDNA3, wt-HA-JNK2, or HA-JNK2-as3 by standard calcium phosphate precipitation methods. Thirty hours later, cells were exposed to UV-C (60 J/m^2) and harvested after 45 min. Protein samples were prepared from cells as previously described (31).

Orthophosphate in Vivo Labeling—Cells were cultured in phosphate-free medium for 1 h before addition of [^{32}P]orthophosphate (1 mCi/plate) for 1 h. HA antibody immunoprecipitated HA-K was separated on SDS-PAGE and transferred onto nitrocellulose membrane following autoradiography and Western blot.

RESULTS

Identification of K Protein as a JNK Substrate Using a Mutant Form of JNK and a Corresponding Form of Modified ATP—To enable selective screening for putative JNK substrates, we modified the ATP-binding site on JNK (JNK analogue specific 3; JNK-as3) so that it utilizes an ATP analogue. Based on the success of engineering both tyrosine and serine/threonine kinases from a wide variety of protein kinase families including, *Src, Fyn, CDK2, Cla4* (yeast Pak kinase), *CaMKII*, and *fus3* (yeast MAPK), we have identified two key residues that must be modified in wild-type kinase to allow recognition of ATP analogues modified at the N (6) position (45–48, 67). These two residues in *JNK* are M108G and L168A, respectively, and are analogous to the mutations required to create Cla4-as3 (Fig. 1; Ref. 68). The mutations M108G and L168A maintained JNK inducibility by UV as well as its substrate recognition (Fig. 2*a*). To identify the modified form of ATP that exhibits the highest affinity to JNK-as3 we compared four forms of ATP, of which N^6-(2-phenythyl)-ATP exhibited the strongest ability to inhibit phosphorylation of c-Jun by modified but not wt forms of JNK when added in excess as the cold form of ATP (Fig. 2*b*). ^{32}P-Labeled N^6-(2-phenythyl)-ATP also exhibited the highest affinity for the mutant but not for the wt form of JNK in its ability to phosphorylate c-Jun (Fig. 2*c*). Given the low cross-reactivity with the wt form of JNK and the high affinity for the modified JNK, these observations suggest that N^6-(2-phenythyl)-ATP could be used to enable detection of specific JNK substrates in the presence of endogenous form of JNK.

To identify proteins specifically phosphorylated by modified JNK, cellular extracts were incubated with modified JNK that had been immunopurified from 293T overexpressing cells in the presence of ^{32}P-labeled N^6-(2-phenythyl)-ATP. Phosphorylated proteins were separated on two-dimensional gels followed by silver staining (Fig. 3*b*) and subsequent autoradiography (Fig. 3*a*). Following alignment of the phosphorylated proteins

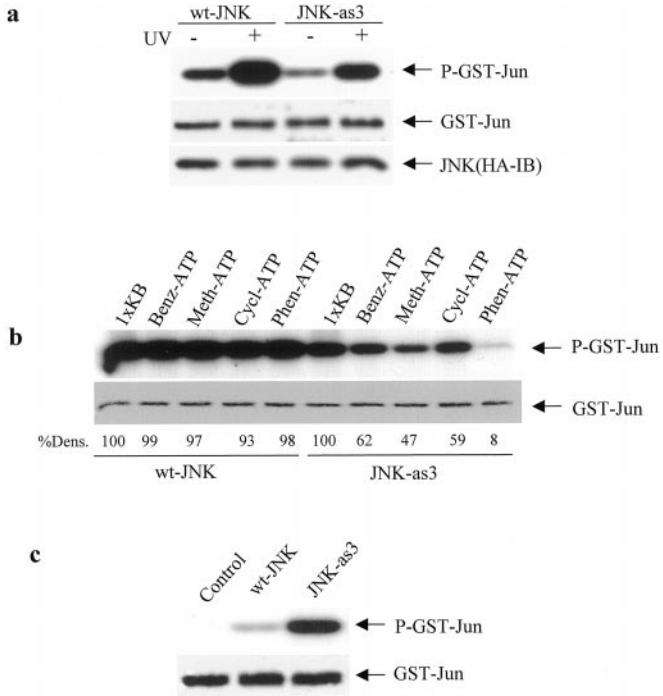

FIG. 2. **Generation of ATP pocket mutant JNK and identification of ATP analogue.** *a, in vitro* kinase assay using GST-Jun as substrate and wt- or JNK-as3 (analog specific 3, mutated on aa M108G,L168A) immunopurified from UV-treated (60 J/m^2) 293T cells was carried out as previously described (33), demonstrating that mutations of JNK did not alter its activation by UV and phosphorylation of c-Jun (*upper panel*). *Lower panel* reveals that equal amounts of immunopurified kinase and substrate were used. *b, in vitro* kinase assay using modified ATP (N^6-(benzyl)-ATP (*Benz-ATP*), N^6-(1-methylbutyl)-ATP (*meth-ATP*), N^6-(cyclopentyl)-ATP (*Cycl-ATP*), and N^6-(2-phenethyl)-ATP) as competitor. Kinase reactions were carried out as above in the presence of 200 μM modified ATP (*upper panel*). *Lower panel* is Coomassie Brilliant Blue (*CBB*)-stained GST-Jun substrate. *c*, *in vitro* kinase assay using ^{32}P-labeled Phen-ATP. Kinase reaction was carried out in the kinase buffer containing GST-Jun substrate, wt-JNK, or JNK-as3 and ^{32}P-Phen-ATP (2 μCi) for 30 min at 30 °C. *Lower panel* is Coomassie Brilliant Blue-stained substrate.

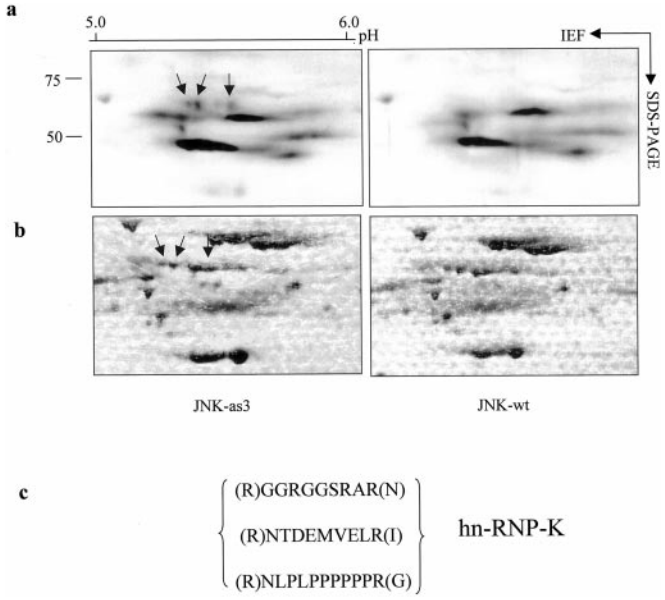

FIG. 3. **Identification of hnRNP-K as JNK substrate.** *a,* separation of JNK-as3-specific substrates. An *in vitro* kinase assay was carried out using protein extracts (100 μg) as substrates in the presence of ^{32}P-Phen-ATP (2 μCi) and JNK-as3 or wt JNK as indicated in the figure followed by two-dimensional PAGE separation using IEF in the first dimension and SDS-PAGE in the second. The figure depicts autoradiograph of the actual kinase reaction. *Arrows* indicate the position of the spots subjected to microsequencing. *b,* reaction performed as indicated in *panel a*, shown is the silver-stained gel with the corresponding spots that were dissected for microsequencing. *c*, outlined are the three peptides identified in microsequencing reaction revealed full homology with the hnRNP-K amino acid sequence.

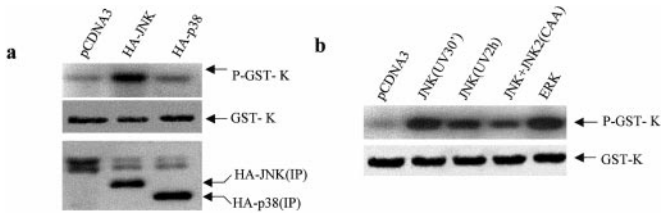

FIG. 4. **hnRNP-K is phosphorylated by JNK or ERK *in vitro*.** *a,* immunokinase assay using bacterially expressed and purified GST-hnRNP K (*GST-K*) fusion protein as substrate and activated forms of HA-JNK or HA-p38 as kinase. 293T cells (*upper panel*) transfected with either HA-JNK or HA-p38 followed by UV irradiation and immunopurification using HA antibodies. *Middle panel* depicts Coomassie Blue-stained substrate. *Lower panel* depicts immunoblot of HA-JNK or HA-p38 that were used for the kinase assay. *b, in vitro* kinase assay using purified GST-K as substrate and endogenous JNK or ERK as kinase that immunopurified from 293T cells using monoclonal anti-JNK1 antibody (PharMingen) or polyclonal anti-ERK2 antibody (Santa Cruz Biotech), respectively (*upper panel*). *Lower panel* represents equal amount of substrate stained with Coomassie Blue.

to those seen in the silver-stained gels, we isolated the corresponding spots. Tandem nanoflow electrospray mass spectrometry of silver-stained spots, which corresponded to the phosphorylated proteins (Fig. 3, *a* and *b*), identified 3 peptide sequences, each of which exhibited 100% homology with hnRNP-K protein (Fig. 3*c*).

Phosphorylation of K Protein by JNK and ERK in Vitro—The full-length cDNA of the K protein was cloned into bacterial and mammalian expression vectors. Bacterially expressed and purified GST-tagged K protein was efficiently phosphorylated *in vitro* by the immunopurified active form of JNK but not by immunopurified p38 (Fig. 4*a*). Further analysis carried out using proteins prepared from cells before and after UV treatment revealed efficient phosphorylation of the K protein as early as 30 min after UV treatment and to lesser degrees by proteins prepared 2 h after UV irradiation (Fig. 4*b*). Forced expression of the constitutively active form of ERK and to a lesser degree of JNK was also efficient in leading to the phosphorylation of K protein (Fig. 4*b*). These observations confirm that the K protein can serve as a substrate for JNK and ERK phosphorylation.

To identify the JNK/ERK phosphoacceptor sites on the K protein, we mutated the proline-driven serines at aa 116, 216, 284, and 353. Of the four mutants, S216A and S353A exhibited the lowest degree of JNK phosphorylation when compared with the wt protein (Fig. 5*a*). When subjected to phosphorylation by ERK, the most efficient decrease in ERK phosphorylation was observed with the S353A mutant. A noticeable decrease was also seen in phosphorylation of the S284A mutant, whereas the S216A mutant revealed a marginal decrease in phosphorylation when compared with the wt form of the K protein (Fig. 5*b*). Of interest is the slight shift in the M_r of the S116A mutant, although the mutation did not affect the degree of phosphorylation by either ERK or JNK (Fig. 5, *a* and *b*). These findings demonstrate that different residues on the K protein may serve as the primary phosphoacceptor sites for JNK *versus* ERK.

Since no single mutant revealed complete loss of phosphorylation of K protein by ERK, we mutated the K protein on multiple phosphoacceptor sites. Among the K proteins that contain different combinations of mutations on different phosphoacceptor sites, a double mutant (S216 and S353) exhibited the most efficient decrease in JNK phosphorylation (Fig. 5*c*),

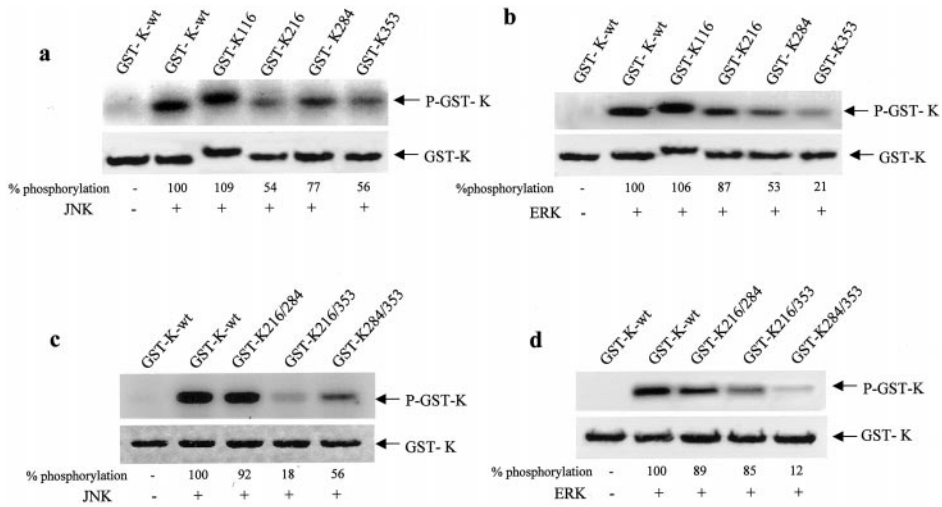

FIG. 5. **Identification of JNK phosphoacceptor sites on the K protein.** *a, in vitro* phosphorylation of phosphomutant GST-K by JNK. GST-K mutated on residues S116A (*GST-K116*), S216A (*GST-K216*), S284A (*GST-K284*), or S353A (*GST-K353*) was produced in bacteria and purified on glutathione beads prior to phosphorylation by immunopurified JNK from UV-treated cells. The *upper panel* depicts the autoradiograph, whereas the *lower panel* shows the Coomassie Blue staining of the substrate used in the reaction. The degree of phosphorylation was quantified on the basis of densitometry scanning. *Numbers* reflects change in % of phosphorylation. *b, in vitro* phosphorylation of single phosphomutant GST-K by immunopurified ERK. The analysis is similar to that described in *panel a*, with the exception that the kinase used in the reaction was ERK immunopurified from MEK-EL expressing cells. *c, in vitro* phosphorylation of double mutant GST-K by JNK. GST-K (mutation on S216A and S284A (*GST-K216/284*), S216A and S353A (*GST-K216/353*), and S284A and S353A (*GST-K284/353*)) were produced in bacteria, purified on glutathione beads and subjected to phosphorylation by JNK. The data shown are representative of at least three experiments. *d, in vitro* phosphorylation of double mutant GST-K by ERK. GST-K (wt or mutants as described in *panel c*) were produced in bacteria, purified on glutathione beads, and subjected to phosphorylation by ERK.

whereas a double mutant (S284A,S353A) exhibited the most efficient decrease in ERK phosphorylation (Fig. 5*d*). A triple mutant (S216A,S284A,S353A) exhibited the same degree of phosphorylation as that shown by the double mutant, suggesting that the primary site for JNK phosphorylation consists of serines 216 and 353 on the K protein. Analysis of secondary structure prediction of wt *versus* phosphomutant forms of the K protein via the predict-protein software did not reveal change in the conformation/structure of the K protein due to these mutations (data not shown).

Phosphorylation of K Protein by JNK and ERK in Vivo—To confirm phosphorylation of the K protein by JNK or ERK *in vivo*, we performed orthophosphate labeling of cells that had been transfected with the K protein and the respective upstream kinases for ERK, p38, or JNK. Immunoprecipitation of the K protein followed by SDS-PAGE and autoradiography revealed efficient phosphorylation upon expression of MEK-EL, a constitutively active form of MEK that drives ERK phosphorylation. UV treatment and JNKK2(CAA) were also capable of mediating phosphorylation of the K protein (Fig. 6).

JNK Phosphorylation of HnRNP K Increases Its Transcriptional Activity—The cellular functions attributed to the K protein include inhibition of RNA translation (55) and transcriptional activation (53, 56–60). Since phosphorylation of the K protein by JNK did not affect its ability to elicit inhibition of RNA translation (49), we have further elucidated the role of JNK in K protein ability to contribute to transcriptional activities. Among promoters that were affected by K protein are those that consist of the AP1 site (53). Given the role of JNK in the activation of c-Jun and ATF2, primary factors that mediate transcription from AP1 sites, we have elucidated the possible contribution of JNK to K protein effect on transcription from AP1 sites.

To this end we have co-transfected Jun-LUC construct with either wt or phosphomutant (216/353) forms of the K protein into 293T cells. To modulate the degree of JNK, activity cells were also transfected with either the constitutive active JNK upstream, kinase (JNKK2(CAA)), or with a dominant negative

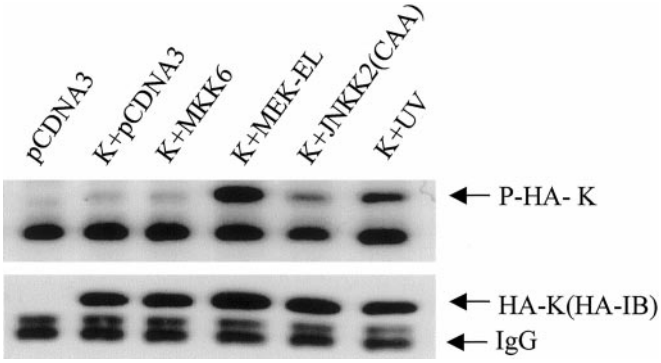

FIG. 6. ***In vivo* phosphorylation of HA-hnRNP K by JNK and ERK.** HA-hnRNP K was co-transfected to 293T cells with or without the upstream kinase of p38, ERK, or JNK (MKK6, MEK-EL, or JNKK2(CAA), respectively) or subjected to UV treatment as indicated in the figure. Orthophosphate labeling was carried out for 2 h prior to protein extraction. ^{32}P-Labeled HA-hnRNP K was immunoprecipitated, washed extensively before separation on SDS-PAGE, transferred to a nitrocellulose membrane, and subsequently analyzed by means of autoradiography (*upper panel*) and Western using antibodies to HA (*lower panel*) to reveal equal loading of the HA-hnRNP-K protein. The *arrows* point to the positions of the K protein, IgG, and a nonspecific HA-cross-reacting band (*ns*).

JNK construct (JNK(APF)) in conjunction with UV treatment as indicated. Whereas forced expression of JNKK2(CAA) alone is sufficient to increase Jun-LUC activity (67%), co-transfection of K and JNKK2(CAA) further induce (>3-fold) Jun-LUC activity (Fig. 7*a*). The latter increase is dependent on K protein phosphorylation by JNK since co-expression of the phosphomutant K protein (K216/353) with JNKK2(CAA) did not increase Jun-LUC activities. The effect of JNK on K proteins ability to facilitate transcription from AP1 site is specific for JNK since the mutant form of JNK (on its phosphoacceptor sites, which render it as a dead kinase and as a dominant negative for endogenous JNK) failed to mediate increase in AP1 activities. Further support for the role of JNK in K protein contribution to AP1-mediated transcription comes from the analysis of Jun-

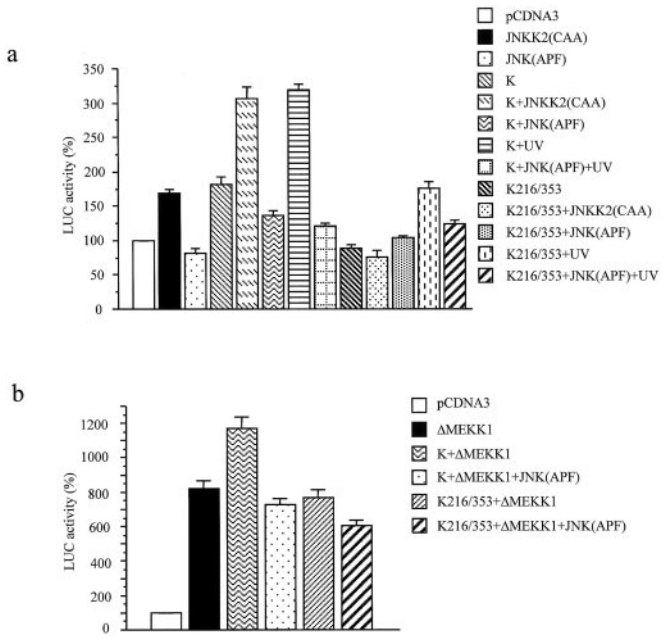

FIG. 7. **JNK phosphorylation of K protein contributes to its transcriptional activities from AP1-bearing promoters.** *a,* Jun-LUC construct (0.1 μg) and wt-k (0.5 μg) or mut-K (k216/353, 0.5 μg) were co-transfected with constitutive active JNK upstream kinase (JNKK2(CAA), 0.5 μg) with dominant negative JNK construct (JNK(APF), 0.5 μg), and with β-galactosidase construct. 24 h after transfection (Gene Jammer into 293 cells) cells were treated with mock or with UV (50 J/m^2). Proteins were prepared 8 h after UV treatment and used for measurement of β-galactosidase activities as well as LUC activities (luciferase measurement kit; Promega). Values shown were normalized per transfection efficiency and represent mean of three experiments. *b,* experiment was performed as described in *panel a*, with the exception that ΔMEKK1 (0.1 μg) was transfected as indicated.

LUC activities in UV-treated cells. Whereas UV irradiation led to a noticeable increase (>3-fold which was comparable to the effect of JNKK2CAA) this increase was completely attenuated upon JNK(APF) expression. This observation suggests that the ability to increase AP1-dependent transcriptional activities via K protein is dependent on JNK activities. A smaller (60%) increase in AP1-mediated transcription was also seen in UV-treated cells that were co-transfected with the phosphomutant form of K, probably due to the effect of UV on the endogenously expressed K protein, which could not be out-competed by exogenous expression of the phosphomutant K form (49). Indeed, co-expression of phosphomutant K and JNK(APF) attenuated the UV-mediated increase in Jun-LUC activities.

Further studies on K protein contribution to transcription from promoters bearing the AP1 site was carried out using the upstream kinase MEKK1 in its constitutively active form (ΔMEKK1). This powerful construct efficiently activates most stress kinase cascades, including JNK, p38, MAPK, and IKK. Forced expression of ΔMEKK1 efficiently activated transcription of Jun-LUC (8-fold), which was further increased (up to 11-fold) upon K protein expression (Fig. 7*b*). Increase observed upon K protein expression was attenuated by JNK-APF. Along these lines, phosphomutant K was not able to augment the transcriptional activities that were mediated by ΔMEKK1. Expression of JNK(APF) in combination with the phosphomutant form of the K protein reduced some of the increase (25%) mediated by ΔMEKK1, probably due to the limited effects of these constructs on the endogenous form of K protein. Together, these findings point to the role of JNK in acquiring K protein ability to contribute to transcriptional activation, as shown here for Jun-LUC bearing promoter sequences.

DISCUSSION

Among the stress-activated protein kinases, JNK has been well characterized as central to the cells decision for life or death in response to most cellular stress-inducing agents. JNK elicits its potent regulatory function through tight regulation of its substrates, which in most cases are bound to JNK and concomitantly targeted by JNK for ubiquitination and degradation under non-stressed conditions. After exposure to any of the diverse stimuli that can activate it, the kinase efficiently phosphorylates the substrate, gaining in stability and activity. Despite the large amount of data accumulated so far with regard to JNKs ability to regulate transcription factors and other stress-related proteins, the number of stress kinases remains small, considering that JNK comprises a family of 3 genes that appear in over 10 different isoforms. It is imperative to identify new JNK substrates that may form part of the stress response and therefore dictate the fate of the stressed cell. Here we demonstrate a new approach to the identification of potential new JNK substrates. Using the technique originally developed by Kevan and colleagues (45–48) for the *Src* gene, we demonstrate the ability to modify the ATP pocket on JNK and consequently to utilize a modified form of ATP that exhibit high affinity toward the modified substrate. This new match enables selective phosphorylation of JNK substrates when the modified kinase and ATP are supplied. Importantly, the modification does not hamper JNK activation by stress, nor does it affect the recognition of JNK substrates, as shown here for c-Jun. Using this approach, the current studies demonstrate the identification of hnRNP-K as a JNK and ERK substrate. The phosphoacceptor sites for JNK and ERK on the K protein are different, and indeed, ERK phosphorylation results in biological consequences different from those of phosphorylation by JNK (49). Whereas ERK phosphorylation on aa 284 and 353 contributes to K protein nuclear export and concomitant inhibition of RNA translation (49), phosphorylation by K protein on aa 216 and 353 increases the transcriptional effects of the K protein. This finding illustrates the diverse forms of regulation of K protein by varying protein kinases, each of which contributes to different K protein functions.

The method developed and employed here for the identification of novel JNK substrates could be employed for identification of JNK substrates in various tissues, in response to different stimuli, at various stages of development, and in promotion and progression of human tumors. Each of the scenarios requires that the set of proteins be used against the corresponding controls to assure that novel JNK substrates are selected. Overall, the altered JNK and its corresponding ATP as described in the current study open new horizons for elucidating novel JNK substrates.

Implications of K protein phosphorylation by JNK are illustrated for K proteins contribution to transcriptional activities, in this case via AP1 sequences. Among the mechanisms underlying K protein ability to confer increased transcriptional output are interconversion of duplex and single-stranded DNA (59) and association with the C/EBPβ (60), each of which could be better affected by the phosphorylated form of the K protein, which may increase affinity to associated proteins or DNA. The increased effect of the K protein on transcription is expected to have a wide effect on transcriptional output due to the general nature of K protein effect on transcriptional regulation.

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