IDENTIFICATION OF NEW JNK SUBSTRATE USING ATP POCKET MUTANT JNK AND A CORRESPONDING ATP ANALogue.

Hasem Habelhah, Kavita Shah¹, Lan Huang², A.L Burlingame², Kevan M. Shokat¹, and Ze’ev Ronai*

Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York NY 10029, ¹Department of Cellular and Molecular Pharmacology, and ²Pharmaceutical Chemistry, UCSF, San Francisco, California 94143.

*Corresponding Author - Ruttenberg Cancer Center, Mount Sinai School of Medicine, 1425 Madison Ave. Rm 15-20, New York NY 10029; Fax 212 849 2425. Email – zeev.ronai@mssm.edu
Abstract

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-phenethyl) ATP, a modified form of ATP that exhibits high specificity and affinity for the modified, but not the wt 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 (hnRNP-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 hnRNP-K mediated transcriptional activities.
INTRODUCTION

The family of mitogen-activated protein kinases (MAPK) 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 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 Thr183 and Thr185 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 2-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 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 (KH) 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 bi-directional 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 KH domain protein, hnRNP-E1, it binds to a CU-rich "differentiation control
element" (DICE) in the 3'UTR of 15-lipoxygenase (LOX) mRNA 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
carboxy 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 interleukin1-responsive K
protein kinase have been shown to phosphorylate hnRNP K; 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 DMEM supplemented with Calf Serum (10%) and antibiotics.

Plasmids. hnRNP K cDNA was PCR amplified using an HA-tagged primer and cloned into pCDNA3 or pGEX-4T-2. Mutations on serine phosphorylation sites (S116A, S216A, S284A, 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 E. coli BL21 and purified using Immobilized glutathione beads (PIERCE) by standard methods.

32P-labeling of phenythyl ADP. E. coli nucleoside diphosphate kinase (NDPK; 0.1mg) was mixed with 100μCi [γ-32P]-ATP (6000Ci/Mmol, Amersham) in HBS (150mM NaCl, 20mM HEPES, pH7.4) containing 5mM MgCl2 and equilibrated at room temperature (RT) for 5min followed by separation on a Bio-spin column (p-6). Phenythyl ADP (5μl of 0.1mM) was added to the phosphorylated NDPK for 10min at RT before the reaction mix was heated (80°C for 2min) and subsequently spun down to pellet the denatured NDPK (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, pH7.4, 0.5mM EGTA, 1mM DTT, 2mM MgCl2, 2mM MnCl2, 5mM NaF, 0.1 mM NaVO3,
5mM β-glycerol phosphate and 75 mM NaCl) as described previously (33). Briefly, 2μg GST-Jun or 100μg lysate were incubated with immunopurified HA-JNK or HA-JNK-as3 in the presence of kinase buffer containing 3μCi \([^{32P}-\gamma]ATP\) or \(^{32P}\)-labeled N\(^6\) (2-phenethyl) ATP and 25μM cold ATP for 30 min at 30°C. Phosphorylated GST-Jun was separated on SDS-PAGE, and phosphorylated lysate was separated by 2D-PAGE followed by staining and autoradiography.

**Two-Dimensional Gel Electrophoresis** Isoelectric focusing (IEF) gels (consist of 8M urea, 4% Acrylamide/bisacrylamide, 2% NP-40, 1.6% pharmalyte pH5-8 and 0.4% pharmalyte pH3-10) were prepared in gel tube (ID 2mm x 18cm; BIO-RAD). Following polymerization (3hrs), IEF gels were pre-run for 1hr at 200V with 10mm H\(_3\)PO\(_4\) as anode butter and 20mm NaOH as cathode buffer. Samples were loaded (40ul) and run at 400 V for 14-16 hrs followed by 800 V for 1hr. IEF gels were removed from tube and equilibrated in SDS sample buffer for 15 min at RT before they were placed on 12% SDS-polyacrylamide gel and run at 40mA until the dye front reached to the bottom of the gel.

**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; NLPPPPPPPR, 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 (60J/m\(^2\)) and harvested after 45min. Protein
samples were prepared from cells as previously described (31).

**Orthophosphate in vivo labeling.** Cells were cultured in phosphate-free medium for 1 hr before addition of $^{32}$p-orthophosphate (1 mCi per plate) for 1 hr. 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 analog 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, fus3 (yeast MAPK), we have identified two key residues that must be modified in wild-type kinase to allow recognition of ATP analogs modified at the N(6) position (45-48, 67) These two residues in JNK are M108G and L168A respectively, are analogous to the mutations required to create Cla4-as3 (Figure 1; 68). The mutations M108G and L168A maintained JNK inducibility by UV as well as its substrate recognition (Figure 2a). 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-phenethyl) 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 (Figure 2b). $^{32}$P-labeled N$^6$(2-phenethyl) ATP also exhibited the highest affinity for the mutant but not for the wt form of JNK in its ability to phosphorylate c-Jun (Figure 2c).
Given the low cross-reactivity with wt form of JNK and the high affinity for the modified JNK, these observations suggest that N^6(2-phenethyl) 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 over-expressing cells in the presence of ^32P-labeled N^6(2-phenethyl) ATP. Phosphorylated proteins were separated on 2D gels followed by silver staining (Figure 3b) and subsequent autoradiography (Figure 3a). Following alignment of the phosphorylated proteins 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 (Figure 3a, 3b), identified 3 peptide sequences, each of which exhibited 100% homology with hnRNP-K protein (Figure 3c).

**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 (Figure 4a). Further analysis carried out using proteins prepared from cells before and after UV-treatment revealed efficient phosphorylation of the K protein early as 30min after UV treatment and to lesser degrees by proteins prepared 2h after UV irradiation (Figure 4b). 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 (Figure 4b). These observations confirm that 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 (Figure 5a). 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 (Figure 5b). Of interest is the slight shift in the MW of the S116A mutant, although the mutation did not affect the degree of phosphorylation by either ERK or JNK (Figures 5a, 5b). These findings demonstrate that different residues on the K protein may serve as the primary phosphoacceptor sites for JNK vs. 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 5c), whereas a double mutant (S284A and S353A) exhibited the most efficient decrease in ERK phosphorylation (Figure 5d). A triple mutant (S216A, S284A, and 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 vs. 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 ortho-phosphate 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 (Figure 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 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 degree of JNK activity cells were also transfected with either the constitutive active JNK upstream, kinase (JNKK2(CAA)) or with a dominant negative 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 (Figure 7a). 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 protein’s ability to facilitate transcription from AP1 site is specific for JNK since 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-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 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 (Figure 7b). 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 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 finding 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 cell’s 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 JNK’s 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 his colleagues for the Src gene (45-48), we demonstrate the ability to modify the ATP pocket on JNK and consequently to utilize a modified form of ATP that exhibit high affinity towards 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 protein’s 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 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.

Acknowledgments

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Figure Legends

Figure 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, 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, are analogous to the mutations required to create Cla4-as3.

Figure 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 (60J/m²) 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 [N⁶-(benzyl)-ATP (Benz-ATP), N⁶-(1-methylbutyl)-ATP (meth-ATP), N⁶-(cyclopentyl)-ATP (Cycl-ATP) and N⁶-(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 $^{32}\text{P}$-labeled Phen-ATP. Kinase reaction was carried out in the kinase buffer containing GST-Jun substrate, wt-JNK or JNK-as3 and $^{32}\text{P}$-Phen-ATP (2µCi) for 30 min at 30°C. Lower panel is CBB stained substrate.

**Figure 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 (100µg) as substrates in the presence of $^{32}\text{P}$-Phen-ATP (2µCi) and JNK-as3 or wt JNK as indicated in the figure followed by 2D-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.

**Figure 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 commassie 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 (SantaCruz) respectively (upper panel). Lower panel represents equal amount of substrate stained with commassie blue.

**Figure 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 commassie 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 3 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.

Figure 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 (M KK6, MEK-EL or JNKK2(CAA) respectively) or subjected to UV-treatment as indicated in the figure. Orthophosphate labeling was carried out for 2h 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 non-specific HA-cross-reacting band (ns).

Figure 7. JNK phosphorylation of K protein contributes to its transcriptional activities from AP1-bearing promoters.
a. Jun-LUC construct (0.1µg) and wt-k (0.5µg) or mut-K (k216/353, 0.5µg) were co-transfected with constitutive active JNK upstream kinase (JNKK2(CAA), 0.5µg) or with dominant negative JNK construct (JNK(APF), 0.5µg) and with β-Gal construct. 24hrs after transfection (Gene Jammer into 293 cells) cells were treated with mock or with UV (50J/m²). Proteins were prepared 8hrs after UV treatment and used for measurement of β–Gal activities as well as LUC activities (luciferase measurement kit; Promega). Values shown were normalized per transfection efficiency and represent mean of 3 experiments.

b. Experiment was performed as described in panel a, with exception that ΔMEKK1 (0.1µg) was transfected as indicated.
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Fig. 2

(a) Western blot analysis showing the phosphorylation of GST-Jun (P-GST-Jun) and GST-Jun levels under different conditions. The blots were probed with antibodies specific to phospho-JNK (P-JNK) and total JNK (JNK).

(b) Densitometric analysis of the bands from panel (a). The densitometric values are shown for wt-JNK and JNK-as3 treatments.

(c) Ablation experiment showing the effect of JNK deletion on the phosphorylation of GST-Jun (P-GST-Jun) and GST-Jun levels.
Fig. 3

(a) Representative 2D gel images showing protein expression.

(b) Magnified view of specific regions indicated by arrows.

(c) Sequences of hn-RNP-K:

\[
\begin{align*}
(R) & \text{GGRGGGSRAR(N)} \\
(R) & \text{NTDEMVELR(I)} \\
(R) & \text{NLPLPPPPPPPPPPR(G)}
\end{align*}
\]
Fig. 4
Identification of a new JNK substrate using ATP pocket mutant ADK and a corresponding ATP analogue
Hasem Habelhah, Kavita Shah, Lan Huang, Alma L. Burlingame, Kevan M. Shokat and Ze'ev Ronai

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