An oncogenic mutation uncouples the v-Jun oncoprotein from positive regulation by the SAPK/JNK pathway *in vivo*

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Stimulation of c-Jun transcriptional activity via phosphorylation mediated by the stress-activated or c-Jun amino-terminal (SAPK/JNK) subgroup of mitogen-activated protein kinases (MAP kinases) is thought to depend on a kinase-docking site (the delta region) within the amino-terminal activation domain, which is deleted from the oncogenic derivative, v-Jun [1–3]. This mutation markedly enhances v-Jun oncogenicity [4,5]; however, its transcriptional consequences have not been resolved. In part, this reflects uncertainty as to whether binding of SAPK/JNK inhibits c-Jun function directly [6,7] or, alternatively, serves to facilitate and maintain the specificity of positive regulatory phosphorylation [8]. Using a two-hybrid approach, we show that SAPK/JNK stimulates c-Jun transactivation in yeast and that this depends on both catalytic activity and physical interaction between the kinase and its substrate. Furthermore, c-Jun is active when tethered to DNA via SAPK/JNK, demonstrating that kinase binding does not preclude transactivation. Taken together, these results suggest that SAPK/JNK acts primarily as a positive regulator of c-Jun transactivation *in situ*, and that loss of the docking site physically uncouples v-Jun from this control. This loss-of-function model accounts for the deficit of v-Jun regulatory phosphorylation and repression of TPA response element (TRE)-dependent transcription observed in v-Jun-transformed cells and predicts that an important property of the oncoprotein is to antagonise SAPK/JNK-dependent gene expression.

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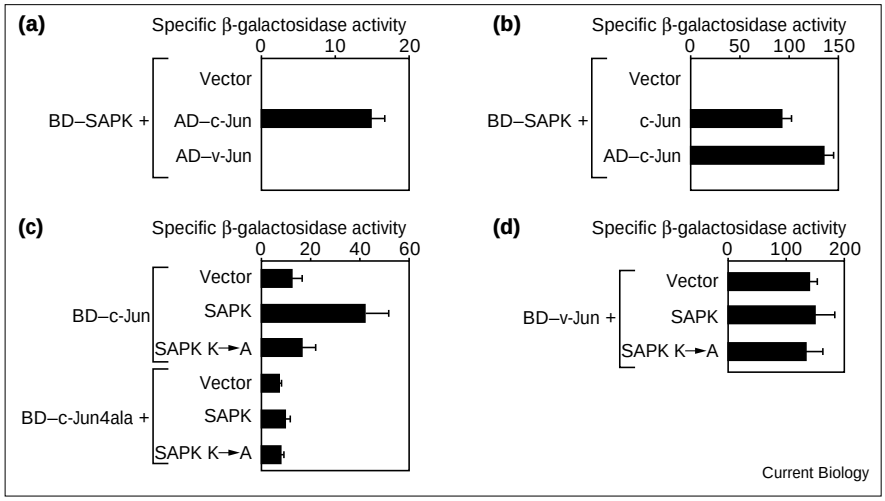
Results and discussion

We fused the 54 kDa isoform of rat SAPKβ (corresponding to human JNK3 [7,9]) to the Gal4 DNA-binding domain (BD–SAPK), and the Gal4 activation domain (AD) to either c-Jun (AD–c-Jun) or a mutant derivative lacking the delta region (AD–v-Jun). Coexpression of AD–c-Jun with BD–SAPK in a yeast strain harbouring a Gal1–*lacZ* reporter resulted in β-galactosidase activity, indicating that protein–protein interactions were occurring (Figure 1a). Several observations indicated that the interaction between AD–c-Jun and BD–SAPK was highly specific. First, no interaction was observed with analogous BD fusion proteins containing the catalytic subunit of protein kinase A (PKA) or ERK2 (data not shown). Second, the delta region was essential for the interaction, as no activity was observed with AD–v-Jun, even though both activators interacted equally well with a BD fusion protein containing the Fra-2 leucine zipper, demonstrating that AD–v-Jun was expressed and capable of activating reporter gene transcription (data not shown).

Because the delta docking site is located within the c-Jun activation domain, a number of studies have speculated that binding of SAPK might mask or inhibit c-Jun transactivation directly [6,7]. To test this, we compared the level of activation mediated by AD–c-Jun with a derivative lacking the Gal4 AD tag. Although weaker than AD–c-Jun, c-Jun alone was able to activate transcription when tethered to DNA via BD–SAPK (Figure 1b), thus ruling out complete masking or inhibition of the activation domain by SAPK. We also fused c-Jun and v-Jun to the Gal4 DNA-binding domain (BD–c/v-Jun), and coexpressed these hybrid activators with wild type or a catalytically inactive mutant of SAPK containing the substitution Lys55→Ala (SAPK K→A [10]). As shown in Figure 1c, SAPK stimulated the transcriptional activity of BD–c-Jun. Although the magnitude of this increase was modest (two- to three-fold in different experiments), it was highly reproducible and dependent on SAPK catalytic activity, as SAPK K→A had no effect. Stimulation also required phosphorylation of c-Jun, as SAPK did not affect the activity of a mutant (BD–c-Jun4ala) in which all of the potential SAPK phosphorylation sites are replaced by alanines. We saw no evidence that the catalytically inactive SAPK K→A mutant inhibited c-Jun transcriptional activity, even though control experiments indicate that it interacts physically with c-Jun as well as does wild-type SAPK (data not shown).

Figure 1



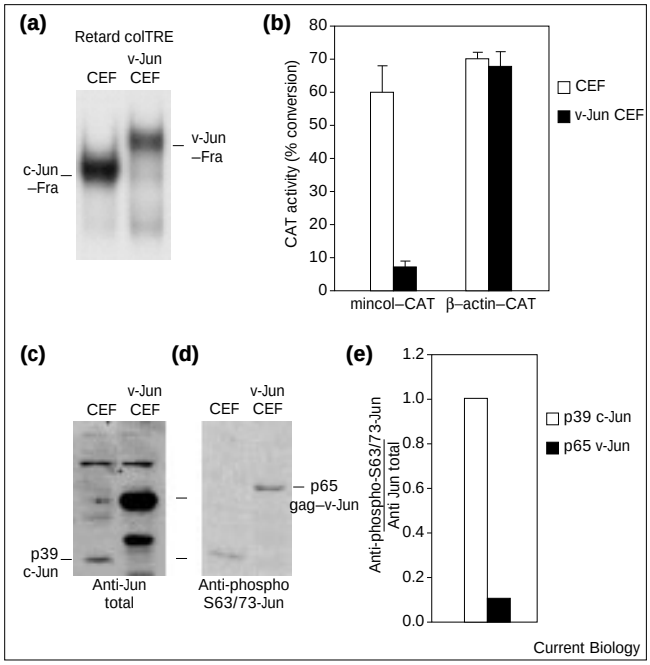
(a) Differential interaction of c-Jun and v-Jun with SAPK *in vivo*. BD–SAPK was expressed (from pGBT9 [25]) together with AD–c-Jun or AD–v-Jun (from pGAD424 [25]) in a yeast strain (SFY526 [25]) harbouring a Gal1–*lacZ* reporter gene. The average and standard deviations of β-galactosidase activity from five independent transformants for each combination are shown. (b) c-Jun activates transcription when tethered to DNA via SAPK. BD–SAPK was expressed (from pGBT9) together with AD–c-Jun or c-Jun lacking the Gal4 activation domain tag (from pMET415 [26]) in SFY526. β-Galactosidase determinations as in (a). (c,d) Interaction with catalytically active SAPK stimulates c-Jun transcriptional activity. BD–c-Jun, a mutant derivative in which all potential SAPK/JNK phosphoacceptor sites (serines 63, 73; threonines 91, 93) are replaced by alanine residues (BD–c-Jun4ala), and BD–v-Jun were expressed (from pGBT9) with SAPK or a catalytically inactive mutant, SAPK K→A (from p415MET25) in SFY526. β-Galactosidase determinations as in (a). DNA segments encoding rat SAPKβ, SAPK K→A, c-Jun, c-Jun4ala and v-Jun were generated by PCR and the authenticity of the resulting constructs verified by sequencing.

In marked contrast, SAPK had no effect on the activity of BD–v-Jun (Figure 1d), even though all of the SAPK/JNK phosphoacceptor sites are conserved in the oncoprotein. We believe that the most likely explanation for this observation is that phosphorylation and thus stimulation of c-Jun transactivation activity by SAPK *in vivo* requires physical association of the kinase with its substrate. As noted by others [11], however, BD–v-Jun is a more potent activator than BD–c-Jun in yeast. Although the reason for this is not known, we do not think that this feature can necessarily be extrapolated to vertebrate cells. First, it is evident only with Gal4–Jun fusion proteins, as native v-Jun does not activate transcription of TRE–*lacZ* reporters more strongly than does c-Jun in yeast (data not shown). Second, the basal activity of BD–c-Jun (and BD–v-Jun) in yeast in the absence of SAPK is largely independent of the modification state of the SAPK/JNK regulatory sites as judged by the effect of multiple alanine substitutions (Figure 1c and data not shown). This contrasts with the situation in vertebrate cells where phosphorylation of these residues is a major determinant of c-Jun transcriptional activity (reviewed in [12]). Taken together, these results indicate that binding of SAPK/JNK does not preclude c-Jun transactivation, but that potentiation of this function by phosphorylation *in vivo* is likely to require physical association of the kinase with its substrate. Because v-Jun does not interact with SAPK, this model predicts that the oncoprotein will exhibit a deficit of regulatory phosphorylation and thus of transcriptional activity in vertebrate cells.

To evaluate this model, we took advantage of the finding that whereas complexes containing the 39 kDa c-Jun protein account for essentially all of the TRE-specific binding activity in normal avian fibroblasts, these are replaced by complexes containing the 65 kDa gag–v-Jun protein after transformation by a retrovirus encoding v-Jun, owing to extinction of endogenous c-*jun* mRNA and protein expression (Figure 2a; [13–15]). Surprisingly, although the overall level of TRE-specific DNA-binding activity is similar, transcription of a transfected reporter plasmid driven by a consensus TRE motif (minimum collagenase gene promoter (mincol)–CAT [16]) is greatly depressed in v-Jun-transformed cells (Figure 2b). This is not due to differences in transfection efficiency, as gene promoters devoid of TRE motifs support similar levels of transcription in both cell backgrounds (Figure 2b). Nor is it attributable to variations in heterodimeric partners, as it has previously been shown that c-Jun and v-Jun are associated with identical Fos-related proteins (Fras) in normal and transformed cells [13].

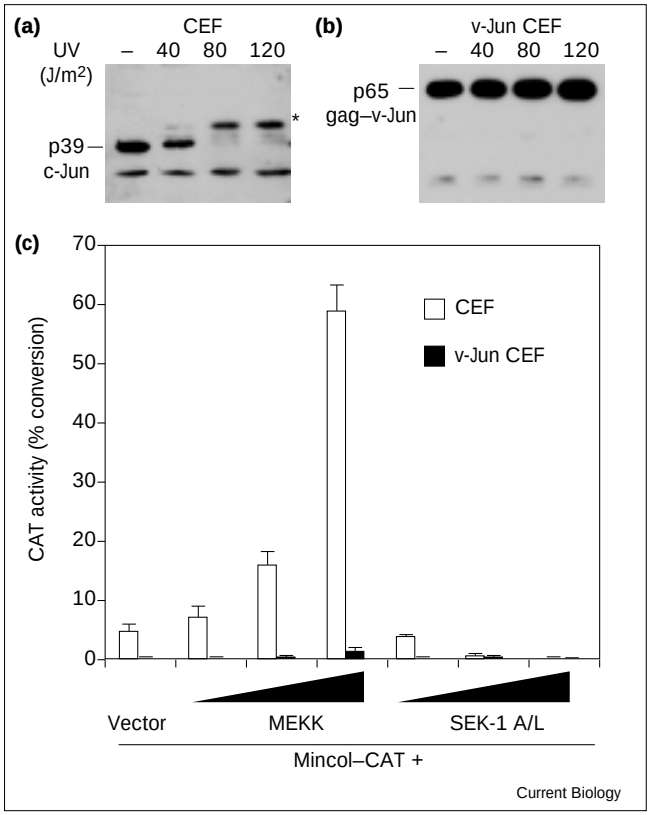
To determine whether defective phosphorylation of the mutant oncoprotein might underlie the reduction in transcriptional activity, we evaluated the modification state of the principal SAPK/JNK regulatory sites (serines 63 and 73; S63/73) in c-Jun and gag–v-Jun. Western blotting analysis (Figure 2c,d) using a non-discriminating anti-Jun antiserum indicated that gag–v-Jun was approximately 20-fold more abundant than c-Jun, but that the mutant protein was detected only very weakly by an antiserum specific for Jun molecules phosphorylated on S63, S73, or both. Quantitation of these data revealed that these sites in gag–v-Jun were underphosphorylated by a factor of approximately 10 compared to the corresponding residues in c-Jun, thus closely approximating the reduction in transcriptional activity (Figure 2e). Differential S63/73 phosphorylation was

Figure 2



(a) Gel retardation analysis of colTRE-specific DNA-binding activity in normal and v-Jun-transformed fibroblasts. Whole cell extracts (15 μg) were analysed by gel retardation using an oligonucleotide containing the consensus TRE from the collagenase gene promoter [16], as described in [13]. Previously characterised c-Jun–Fra and v-Jun–Fra complexes [13] are indicated. (b) TRE-dependent transcription is repressed in v-Jun-transformed cells. Reporter plasmids containing the chloramphenicol acetyl transferase (CAT) gene under the control of either the minimum collagenase gene promoter (mincol–CAT) or the human β-actin gene promoter (β-actin–CAT) were transfected into cultures of normal fibroblasts (CEF) or v-Jun-transformed fibroblasts (v-Jun CEF), and CAT assays performed as described previously [13]. Values represent the average and standard deviations of five independently transfected dishes. (c–e) Differential phosphorylation of the S63/73 SAPK/JNK regulatory phosphorylation sites in c-Jun and v-Jun *in vivo*. (c,d) Western blotting analysis of c-Jun and gag–v-Jun in 100 μg whole cell extracts using either a non-discriminating anti-Jun antiserum (anti-Jun total [13]), or a polyclonal antiserum that recognises only Jun molecules phosphorylated on serines 63, 73, or both (anti-phospho S63/73-Jun). (e) The relative staining intensity of c-Jun and gag-v-Jun using the non-discriminating anti-Jun total and anti-phospho S63/73-Jun antisera was quantitated by densitometry (the ratio for c-Jun was arbitrarily set at one).

Figure 3



(a,b) Amino-terminal phosphorylation of v-Jun is non-inducible *in vivo*. (a) Normal or (b) v-Jun-transformed fibroblasts were exposed to the indicated dose of UV irradiation and extracts prepared after 15 min. After western blotting, the Jun proteins were visualised using the non-discriminating anti-Jun antiserum [13]. Increased amino-terminal phosphorylation of c-Jun results in electrophoretic retardation (asterisk). A fivefold shorter exposure (of the same blot) is presented for the v-Jun-transformed cell samples to compensate for the higher expression of gag–v-Jun. (c) Modulation of TRE-dependent transcription in normal and v-Jun-transformed cells by catalytic activators or inhibitors of SAPK/JNK. The indicated cell cultures were transfected with 3 μg of the mincol–CAT reporter [16] together with increasing amounts (3, 6, and 12 μg) of expression vectors encoding either MEKK (from pCMV5 [27]) or SEK-1 A/L (from pRcCMV [20]). Control experiments have shown that the effects of MEKK and SEK-1 A/L on transcription are dependent on the presence of a TRE motif in the reporter plasmid (S.H., A.K. and D.A.F.G, unpublished observations).

also evident when c-Jun and gag–v-Jun were captured by microscale affinity purification using a biotinylated collagenase promoter TRE (colTRE) oligonucleotide [17], ruling out the possibility that the small amount of phosphorylated v-Jun was selectively bound to DNA (data not shown).

Ultraviolet (UV) light is a potent agonist of SAPK/JNK [9,18], and exposure of normal fibroblasts to increasing doses of UV leads to the appearance of isoforms of c-Jun that migrate more slowly on polyacrylamide gels and are diagnostic of increased amino-terminal phosphorylation ([13]; Figure 3a,b). In contrast, UV exposure does not induce any corresponding modification of gag–v-Jun in transformed fibroblasts, indicating that the low level of v-Jun phosphorylation cannot be augmented by agents that activate the SAPK/JNK pathway *in vivo*. Importantly, these effects are not attributable to fusion with retroviral gag sequences, as an identical deficit in both basal and induced phosphorylation is observed in cells transformed by v-Jun alone (data not shown). As phosphorylation of c-Jun is thought to be an important mechanism through which the SAPK/JNK pathway stimulates TRE-dependent transcription [12], it was of interest to determine whether this signal transduction process was disturbed in v-Jun-transformed cells.

To manipulate SAPK/JNK activity *in vivo*, we transfected increasing amounts of plasmids encoding MEKK, a potent activator of the SAPK/JNK pathway [19], or, alternatively, a catalytically inactive mutant of the SAPK/ JNK upstream activator SEK-1 (SEK-1 A/L [20]), together with the mincol–CAT reporter into normal or v-Jun-transformed cells. As predicted, cotransfection of MEKK led to a substantial dose-dependent stimulation of TRE-dependent transcription in normal cells, while the inhibitory SEK-1 A/L mutant led instead to a progressive reduction in activity (Figure 3c). In contrast, MEKK induced only a very small increase in reporter transcription in the v-Jun-transformed cell background, whereas SEK-1 A/L had no significant effect on basal activity (Figure 3c). Thus, TRE-dependent transcription is not only reduced overall in v-Jun-transformed cells, but is also rendered markedly less sensitive to induced fluctuations in the activity of the SAPK/JNK pathway.

Taken together, our results indicate that SAPK acts as a positive regulator of c-Jun transcriptional activity *in situ*, and that deletion of the delta docking region confers a loss-of-function phenotype by uncoupling v-Jun from this regulation. This conclusion challenges the long-standing assumption that v-Jun represents a 'super-activated' form of c-Jun [21,22], as it predicts that v-Jun acts as a 'dominant negative' mutant that will block or antagonise SAPK/JNK-regulated gene expression [23], much as the thyroid hormone receptor derived v-ErbA oncoprotein retains the capacity to bind to DNA but can no longer act as an effector of hormone-regulated transcription [24]. Interestingly, the SAPK/JNK pathway is activated strongly by chemical and radiant stresses likely to induce growth arrest, cell death or both, but is activated only weakly by mitogens [9,18]. We therefore suggest that repression of growth-inhibitory or pro-apoptotic genes may be more closely linked to v-Jun-mediated oncogenesis than is activation of growth-stimulatory genes, as previously supposed.

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