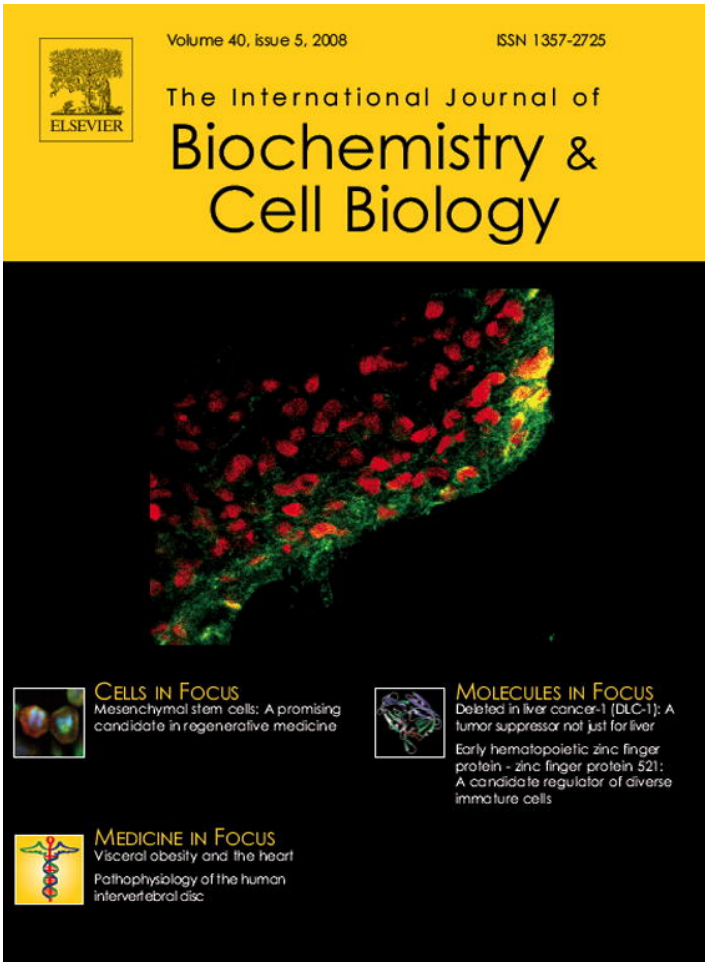

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# Requirements for PKC-augmented JNK activation by MKK4/7

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**Abstract**

The c-Jun N-terminal kinases (JNKs) are activated in response to stress, DNA damage, and cytokines by MKK4 and MKK7. We recently demonstrated that PKC can augment the degree of JNK activation by phosphorylating JNK, which requires the adaptor protein RACK1. Here we report on the conditions required for PKC-dependent JNK activation. In vitro kinase assays reveal that PKC phosphorylation of JNK is not sufficient for its activation but rather augments JNK activation by canonical JNK upstream kinases MKK4 or MKK7 alone or in combination. Further, to enhance JNK activity, PKC phosphorylation of JNK should precede its phosphorylation by MKK4/7. Inhibition of PKC phosphorylation of JNK affects both early and late phases of JNK activation following UV-irradiation and reduces the apoptotic response mediated by JNK. These data provide important insight into the requirements for PKC activation of JNK signaling.


*Keywords:* JNK; MKK4; MKK7; PKC; RACK1

## 1. Introduction

The c-Jun N-terminal kinases (JNKs) are among the major subgroups of mitogen-activated protein kinases (MAPKs) that are induced by diverse signals including growth factors, cytokines, tumor promoters, UV radiation, and hormones. Similar to other MAPKs, JNKs are regulated by a multi-tier module of protein kinases (Karin, 1995). Several MAP kinase kinase kinases (MAP3Ks) activate two distinct MAP kinase kinases, MKK4 and MKK7, which in turn phosphorylate JNK on the TPY motif (Thr183 and Tyr185) within the activation loop leading to their activation (Davis, 2000). In addition to the effects of its canonical upstream kinases and the adaptor family proteins JIP (Whitmarsh, 2006), the degree of JNK activity is affected by diverse signaling pathways including the Protein Kinase C (PKC) and Extracellular-Regulated Kinase (ERK) signaling cascades (Lopez-Bergami et al., 2005, 2007).

The degree of JNK activation in response to diverse stimuli can be augmented by its phosphorylation via PKC (Lopez-Bergami et al., 2005; Liu et al., 2006). JNK activation by PKC is required for its maximal induction by diverse stimuli including cytokines (e.g., tumor necrosis factor alpha, TNFα) and external stress (e.g., UV-irradiation). PKC phosphorylation of JNK on Ser129 augments JNK activation by MKK4/7 (Lopez-Bergami et al., 2005). PKCs effect on JNK requires RACK1 (Receptor for Activated Kinase 1), a 7 WD40 repeat scaffold protein implicated in PKC and Src signaling (Ron et al., 1994). Changes in activity or levels of the

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PKC-RACK1 module affect the magnitude of JNK activation (Lopez-Bergami et al., 2005). ERK contributes to JNK signaling via its ability to upregulate transcription and stability of c-Jun, which in turn induces transcription of RACK1, thereby amplifying the degree of JNK phosphorylation by PKC (Lopez-Bergami et al., 2007).

Activation of JNK causes concomitant activation of its substrates, including c-Jun, ATF2, p53 and Bad, which play key roles in the cellular response to stress and the cell's ability to undergo apoptosis (Davis, 2000) via the transcription of a wide set of genes that affect cell cycle, proliferation, differentiation and death (Shaulian & Karin, 2002; Vogt, 2001).

JNK has been shown to elicit both positive and negative effects on tumor development (Kennedy & Davis, 2003). JNK activation is required for Ras-mediated transformation (Nielsen, Thastrup, Bottzauw, Jaattela, & Kallunki, 2007) and has been found to mediate proliferation and tumor growth (Cui et al., 2006; Yang et al., 2003). These observations are consistent with the finding of constitutively active JNK in tumor samples and derived cell lines (Kennedy & Davis, 2003; Khatlani et al., 2007; Lopez-Bergami et al., 2007). On the other hand, studies using $JNK^{-/-}$ fibroblasts revealed that JNK is not required for Ras-dependent tumor development in vivo and that $JNK^{-/-}$ fibroblast are more tumorigenic than wild-type cells (Kennedy et al., 2003). Along these lines, JNK can elicit both pro-apoptotic and anti-apoptotic functions (Liu & Lin, 2005). Among the parameters that affect JNK function are the cell type, the degree of its activation, the duration of its signal, the expression of its substrates and the activity of parallel signaling pathways, including the PKC and ERK cascades. Those are tissue and cell type specific and are specifically altered in pathological cases.

JNK protein kinases are encoded by three genes, *Jnk1*, *Jnk2*, and *Jnk3* (Davis, 2000). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is primarily expressed in brain, heart, and testis (Bogoyevitch, 2006). Targeted ablation of each of these genes resulted in a different phenotype (Bogoyevitch, 2006; Jaeschke et al., 2006), suggesting that individual JNK isoforms may serve different functions. For example, mutant JNK1 (but not JNK2) mice exhibit a higher incidence of skin and intestinal tumors (She, Chen, Bode, Flavell, & Dong, 2002; Tong et al., 2007), and cells of JNK2 mutant mice exhibit a greater proliferation capacity and levels of c-Jun expression and activity (Sabapathy et al., 2004). Along this line, it has been shown that JNK1, but not JNK2, is essential for TNF-α-induced c-Jun kinase activation, c-Jun expression, and apoptosis (Habelhah et al., 2004). More recent studies using mice expressing a mutant form of JNK that can be selectively inhibited by a chemical compound identified greater similarities among the JNK isoforms (Jaeschke et al., 2006). These studies clearly establish the diversity and complexity of JNK regulation and function.

An important factor that is expected to play a central role in JNKs ability to elicit each of its diverse biological functions relates to the nature of the activating stimulus. Consistent with this expectation, the two MKKs that activate JNK have different preferences for phosphoacceptor sites (Tyr185 for MKK4 and Thr183 for MKK7) (Fleming et al., 2000; Lin et al., 1995). Further, each MKK is selectively regulated by specific extracellular stimuli. Whereas TNF-α and IL-1α preferably activate the MKK7 isoforms, UV-C irradiation and anisomycin cause greater activation of MKK4 (Tournier et al., 2001). Although both MKK4 and MKK7 may be required for maximal activation of JNK, the differential phosphorylation of JNK by MKKs provides the molecular basis for differential activation of JNK by various stimuli (Lin, 2003). Following our finding that PKC contributes another layer to the complex regulation of JNK, we set to examine whether PKC augments MKK4 or MKK7 preferentially, and to directly assess PKC's contribution to JNK activity in vitro and in vivo.

## 2. Materials and methods

### 2.1. Cell lines, reagents and transfection

SW1 and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%) and antibiotics. Cells were transfected using calcium phosphate or LipofectAMINE PLUS Reagent (Invitrogene) following the manufacturer's protocol. Antibodies and reagents were purchased as follows: anti-RACK1 antibody (Ab) (Transduction Technologies) anti-JNK Ab (Santa Cruz), anti-MKK7 Ab (Cell Signaling), anti-MKK4 Ab (Cell Signaling), anti-HA Ab (12CA5, Zymed) and anti-Flag Ab (Sigma). Final concentration of Go6976 (Calbiochem) was 3 μM. When Go6976 was used, control reactions employed with DMSO.

### 2.2. Constructs

Constructs encoding Flag-JNK2, FLAG-JNK2-S129A, FLAG-JNK2-APF, GST-$Jun^{1-89}$, His-JNK2, His-RACK1 and CA- PKCβII were previously described (Fuchs, Dolan, Davis, & Ronai, 1996; Lopez-Bergami et al., 2005).

### 2.3. Protein purification

His-JNK1, His-JNK2 and GST-Jun$^{1-89}$ were affinity purified from bacterial cultures as described (Fuchs & Ronai, 1999). Plasmids expressing FLAG-JNK2, FLAG-MKK4 and FLAG-MKK7 were transfected into HEK293T cells and the FLAG-tagged proteins were affinity purified from the lysates. Briefly, the lysates were incubated with FLAG-beads and washed and the proteins eluted by addition of 1 mg/ml of FLAG peptide (DYKDDDDK, Sigma). The purity of the eluates was assessed by SDS-PAGE and coomassie-blue staining (Suppl. Fig. 1).

### 2.4. JNK coupled kinase assay

His-JNK1, His-JNK2 or FLAG-JNK2 were incubated with FLAG-MKK4 or FLAG-MKK7 in 30 μl of kinase buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 0.5 mM EGTA) plus 25 μM cold ATP for 30 min at 30 °C unless otherwise indicated. When indicated, 18 nM of a catalytically active fragment of rat brain PKC (PKM, from different PKC isoforms, BioMol, PA) and 16 nM of His-RACK1 were added. Time course experiments allowed to determine that the reaction was completed within 15–20 min (Suppl. Fig. 2). Incubations longer than 30 min led to an increase in JNK background activity (data not shown). The reaction was followed by addition of 1 μg of GST-Jun$^{1-89}$ and 2 μCi [$^{32}$P]ATP and incubated for 20 min at 30 °C in a volume of 40 μl. Reaction mixtures were then separated on SDS-PAGE and transferred to a nitrocellulose membrane, and the phosphorylation state of GST-Jun$^{1-89}$ was detected and quantified using a phosphorimager. Under these conditions the rate of activation of JNK was linear with time and enzyme concentration. The membranes were then used for Ponceau S staining and immunoblotting. Immunokinase reactions were performed as described above following immunoprecipitation of endogenous JNK.

### 2.5. RNA interference

Downregulation of MKK4 and MKK7 expression was obtained by transfection of the corresponding SMARTpool siRNA reagents (Dharmacon). To downregulate JNK expression a pair of oligonucleotides containing 19 bp of mouse JNK (AGAATGTCCTACCTTCTCT) were cloned into a pRetroSuper (pRS) vector as described (Lopez-Bergami et al., 2005) and stably transfected into SW1 cells.

### 2.6. Immunoblotting and immunohistochemistry

Cells were harvested and lysed in TNE buffer (20 mM Tris–HCl, pH 7.5, 350 mM NaCl, 1.0% NP-40, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 50 mM NaF, 1.0 mM sodium vanadate, 10 mM β-glycerolphosphate and 1× cocktail protease inhibitors) for 30 min on ice followed by centrifugation at 12,500 × *g* for 20 min at 4 °C. Protein samples (50–100 μg protein) were resolved on 10% SDS-PAGE and transferred to nitrocellulose. The primary antibodies were used at dilutions of 1:500 to 1:3,000. The secondary antibodies were anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:5,000). Signals were detected using ECL (Amersham Life Sciences, NJ).

### 2.7. Apoptosis analysis

Cell cycle distribution was assessed using both propidium iodide (PI) staining (Ivanov et al., 2001) and the TACS Annexin V-PI Apoptosis Detection Kit (R&D Systems).

### 2.8. Statistical analysis

All data are from ≥3 independent experiments and given as mean ± S.E.M. ANOVA was used to determine statistical significance. The null hypothesis was rejected at $p < 0.05$.

## 3. Results

We have demonstrated that JNK activation can be augmented upon its phosphorylation by PKC, which requires the adaptor protein RACK1 (Lopez-Bergami et al., 2005). This contribution of PKC to JNK activation was observed following stimuli known to activate both PKC and JNK (i.e. UV-irradiation, TPA). To gain better knowledge of how PKC affects JNK activity, we studied JNK activation using in vitro protein kinase assays. These assays employ bacterially expressed GST-c-Jun as a substrate and His-JNK1 or His-JNK2, which were also purified from bacterial cultures. Since JNK upstream kinases are activated in a stimuli-dependent fashion, MKK4 and MKK7 were immunopurified from transfected HEK293T cells following UV and TNFα treatment, respectively. We first compared activation of JNK1 and JNK2 by using non-limiting amounts of both MKK4 and MKK7. This experiment revealed that JNK2 was more active than JNK1 in this in vitro system (Fig. 1A). Also, MKK4 was more efficient in activa-

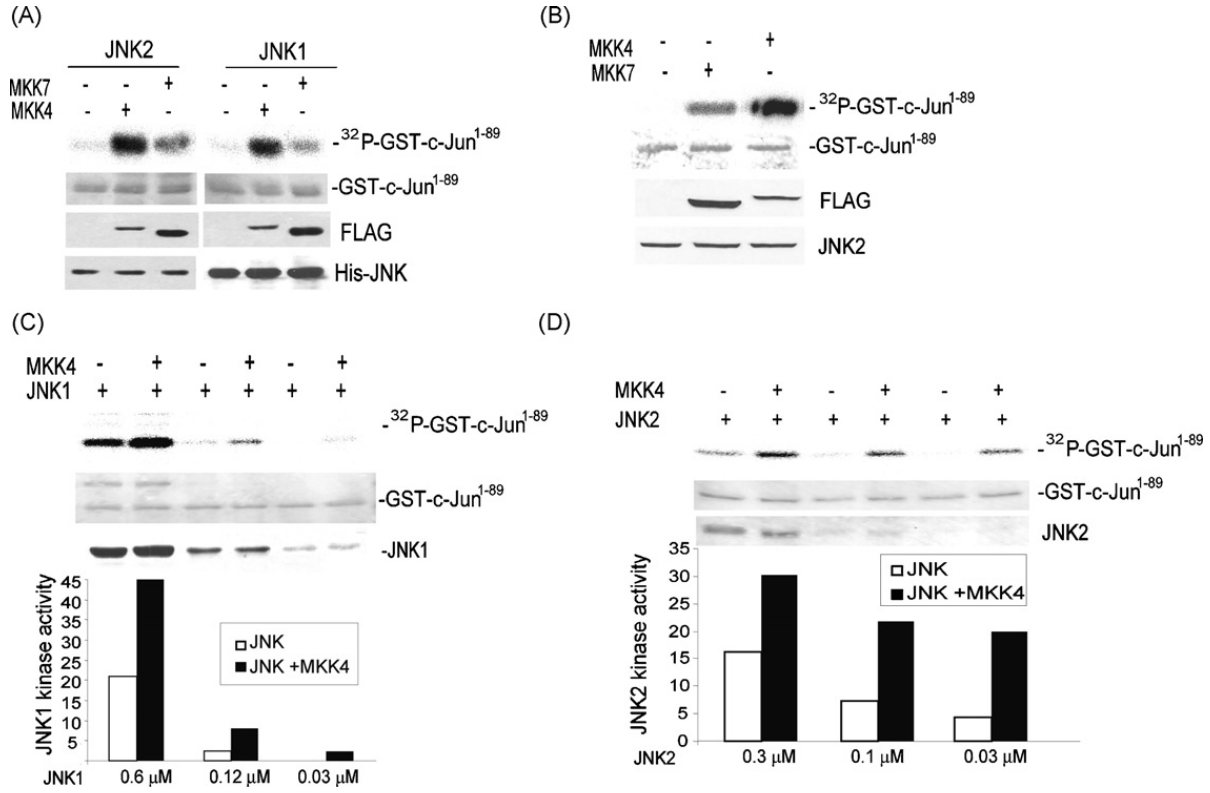


Fig. 1. Activation of JNK1 and JNK2 by MKK4 and MKK7. (A) JNK2 is more active than JNK1 in vitro. His-JNK1 (0.3 μM) or His-JNK2 (0.12 μM) were incubated with FLAG-MKK4 (1.2 μM) or FLAG-MKK7 (3.6 μM) and a coupled kinase reaction was performed as described in Section 2. (B) MKK4 activates JNK2 more efficiently than MKK7. A coupled kinase reaction was performed as described in A except that 0.3 μM of FLAG-JNK2 was used. (C) Dose–response activation of JNK1 by MKK4. The indicated amounts of His-JNK1 were incubated with 0.3 μM of FLAG-MKK4. (D) Dose–response activation of JNK2 by MKK4. The indicated amounts of FLAG-JNK2 were incubated with 0.3 μM of FLAG-MKK4.

tion of both JNK1 and JNK2, compared with MKK7 (Fig. 1A). The lower degree of JNK activation by MKK7, compared with MKK4, was also observed using affinity-purified JNK2 from HEK293T cells (Fig. 1B). These data confirm the role of MKK4/7 in activating JNK while pointing out that in vitro MKK4 is more potent compared with MKK7 and that JNK2 is more active than JNK1.

Next, we assessed activation of JNK by PKC in the presence of either MKK4 or MKK7. To this end, we first established the optimal amounts and molar ratios of the components of the reaction. As shown in Fig. 1C and D, we determined that relatively low amounts of JNK1 and JNK2 (between 0.03 μM and 0.12 μM for JNK1 and between 0.03 μM and 0.1 μM for JNK2) yielded a higher-fold increase in JNK activity in the presence of MKK4. Higher amounts of JNK1 and JNK2 increased JNK activity but also resulted in a higher non-specific background.

PKC has been recently identified as a positive regulator of JNK via phosphorylation of S129 (Liu et al., 2006; Lopez-Bergami et al., 2005) (Fig. 2A). However, this event by itself is not sufficient to activate JNK (see below). Therefore, to further study the mechanism of PKC-dependent JNK activation, we assessed the degree of JNK activation by PKC in the presence of MKK4 and MKK7 using the in vitro kinase assay. Since the goal of this study is to gain further insight into the mechanism of JNK activation by activated kinases (MKKs and PKC) and not activation of these kinases, for the in vitro experiments we used a catalytic fragment of PKC known to bind RACK1 (Stebbins & Mochly-Rosen, 2001). To determine the optimal conditions required for PKC's effect on JNK, we utilized different molar ratios of MKK4/JNK. The adaptor protein RACK1, which bridges PKC and JNK, was included in all of these reactions. Incubation of PKC together with MKK4 activated JNK more efficiently than MKK4 alone (Fig. 2B). Moreover, the effect of PKC on JNK was more evident when using low molar ratios of MKK4/JNK (i.e., 0.5) (Fig. 2B). Higher MKK4/JNK ratios increased the degree of JNK activation while decreasing the effect of PKC on JNK activity (Fig. 2B), consistent with more JNK molecules being phosphorylated by MKK4 alone. Similarly, low MKK7/JNK molar ratios were also more efficient in mediating PKC-dependent JNK activation (Figs. 2D, 3A, 3B and 4A and data not shown). These

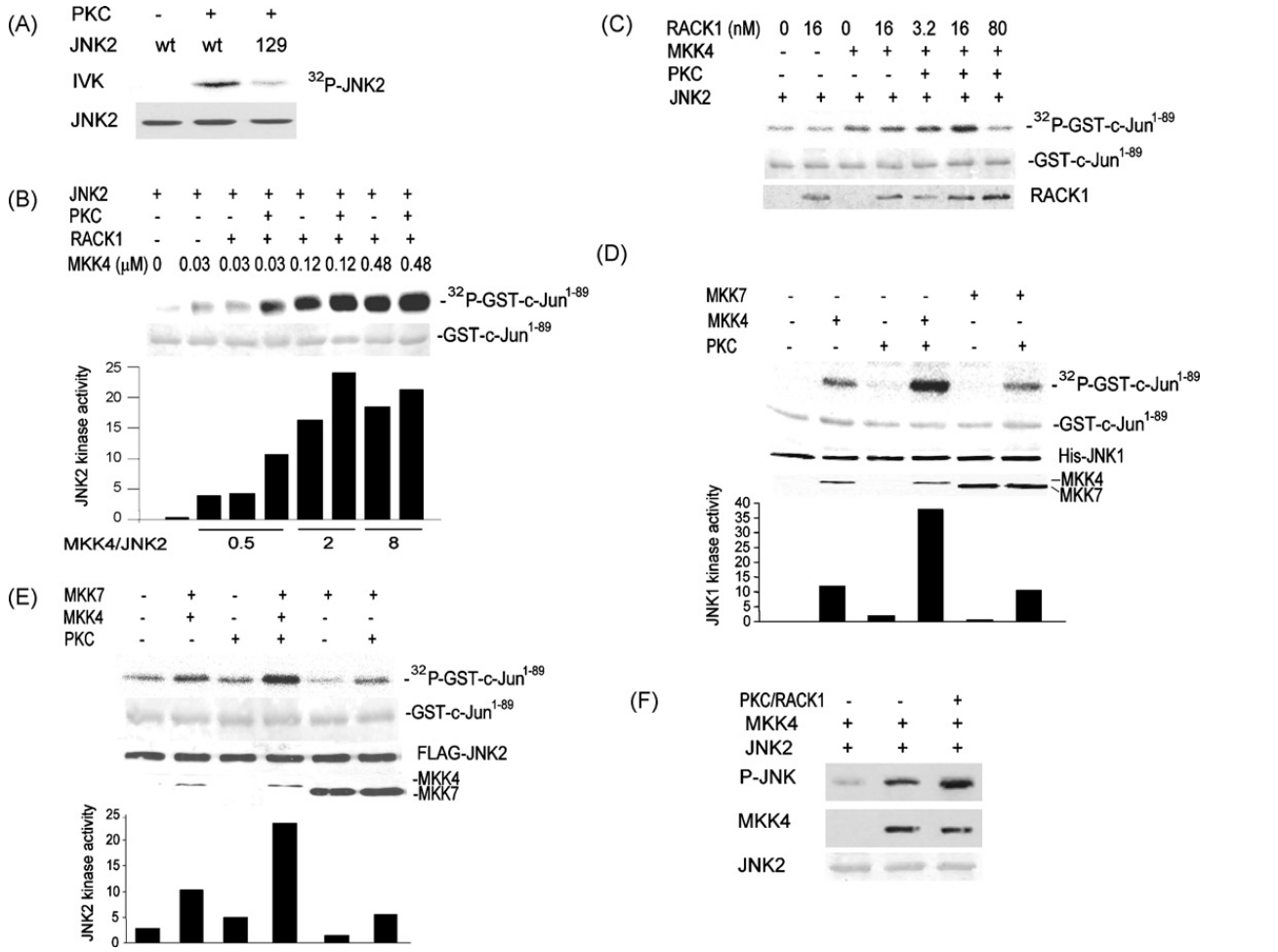


Fig. 2. PKC and MKK4/MKK7 have an additive effect on in vitro JNK activation. (A) PKC phosphorylates JNK at S129. An in vitro kinase reaction (IVK) was performed using active PKC (BioMol) as a kinase and His-JNK2 (1.2 μM) or His-JNK2-S129A (129) as substrates. (B) Increased activation of JNK2 by PKC and MKK4. Flag-JNK2 (0.06 μM) was incubated with the indicated amounts of FLAG-MKK4, His-RACK1 (16 nM) and active PKC (18 nM). JNK activity was assessed as described in Section 2. (C) RACK1 is required for PKCs effect on JNK. FLAG-JNK2 (0.06 μM) was incubated with the indicated amounts of His-RACK1, FLAG-MKK4 (0.03 μM) and active PKC (18 nM). JNK activity was assessed as described in Section 2. (D) PKC and MKK4/MKK7 have an additive effect on JNK1 activation. His-JNK1 (0.06 μM) was incubated with FLAG-MKK4 (0.03 μM), FLAG-MKK7 (0.1 μM), His-RACK1 (16 nM) or active PKC (18 nM). JNK activity was assessed as described in Section 2. (E) PKC and MKK4 have an additive effect on JNK2 activation. Flag-JNK2 (0.06 μM) was incubated with FLAG-MKK4 (0.03 μM), FLAG-MKK7 (0.1 μM), His-RACK1 (16 nM) or active PKC (18 nM). JNK activity was assessed as described in Section 2. Similar results were obtained in three separate experiments. (F) PKC increases JNK phosphorylation by MKK4. Flag-JNK2 (0.5 μg) was incubated with His-RACK1 (40 nM), active PKC (45 nM) and FLAG-MKK4 (0.2 μM) as indicated. After 30 min the reaction was stopped and the degree of JNK phosphorylation was assessed by immunoblot using an antibody against phosphorylated residues T183/Y185.

results suggest that PKC cooperates with MKK4 and MKK7 to activate JNK.

The experiment in Fig. 2B also showed that a PKC-dependent increase in JNK activity requires the presence of RACK1. Interestingly, we also observed that an excess of RACK1 can inhibit the reaction (Fig. 2C), presumably by sequestering either PKC or JNK. Although PKC efficiently phosphorylates JNK (Fig. 2A), incubation of JNK with PKC alone resulted in limited activation of either JNK1 or JNK2 (Fig. 2D and E). This finding indicates that PKC phosphorylation of JNK is not sufficient for its activation. However, when PKC was incubated with JNK1 or JNK2 in the presence of either MKK4 or MKK7, it increased JNK activity substantially over the levels achieved by MKK4 or MKK7 alone (Fig. 2D and E). The fold increase in JNK activation triggered by PKC in the presence of MKK4/7 was 3.1 and 5.2 for MKK4 and MKK7, respectively when using JNK1, and 2.2 and 4.9, respectively, when using JNK2 (Fig. 2D and E). Moreover, the degree of MKK4-dependent JNK phosphorylation increased in the presence of PKC (Fig. 2F and Suppl. Fig. 3). These results provide direct evidence that PKC phosphorylation of JNK augments MKK4/MKK7-dependent activation.

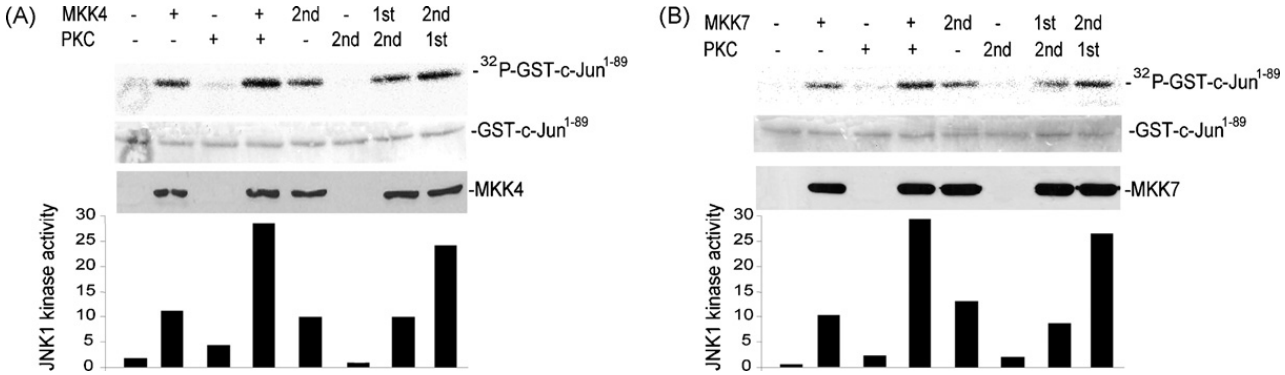


Fig. 3. Higher JNK activation is achieved when PKC phosphorylation precedes phosphorylation by MKK4/7. (A) PKC phosphorylation of JNK must occur prior to MKK4 phosphorylation to enhance JNK activity. His-JNK1 (0.06 μM) was incubated with FLAG-MKK4 (0.03 μM), His-RACK1 (16 nM) or active PKC (18 nM) for 30 min (lanes 1–4). In lanes 7 and 8 sequential activation of JNK by MKK4 and PKC was performed. In lane 7, PKC was added 15 min after addition of MKK4. The reaction was stopped after an additional 15 min (total duration of the reaction was 30 min). In lane 8, MKK4 was added 15 min after addition of PKC. In lanes 5 and 6, JNK was incubated with MKK4 and PKC, respectively for just 15 min. Following incubation of JNK with its upstream kinases as described above, changes in JNK activity were assessed by a kinase reaction using c-Jun as a substrate. (B) PKC phosphorylation of JNK must occur prior to MKK7 phosphorylation to enhance JNK activity. The experiment was performed as described in A but FLAG-MKK7 (0.1 μM) was used instead of FLAG-MKK4. Similar results were obtained in three separate experiments.

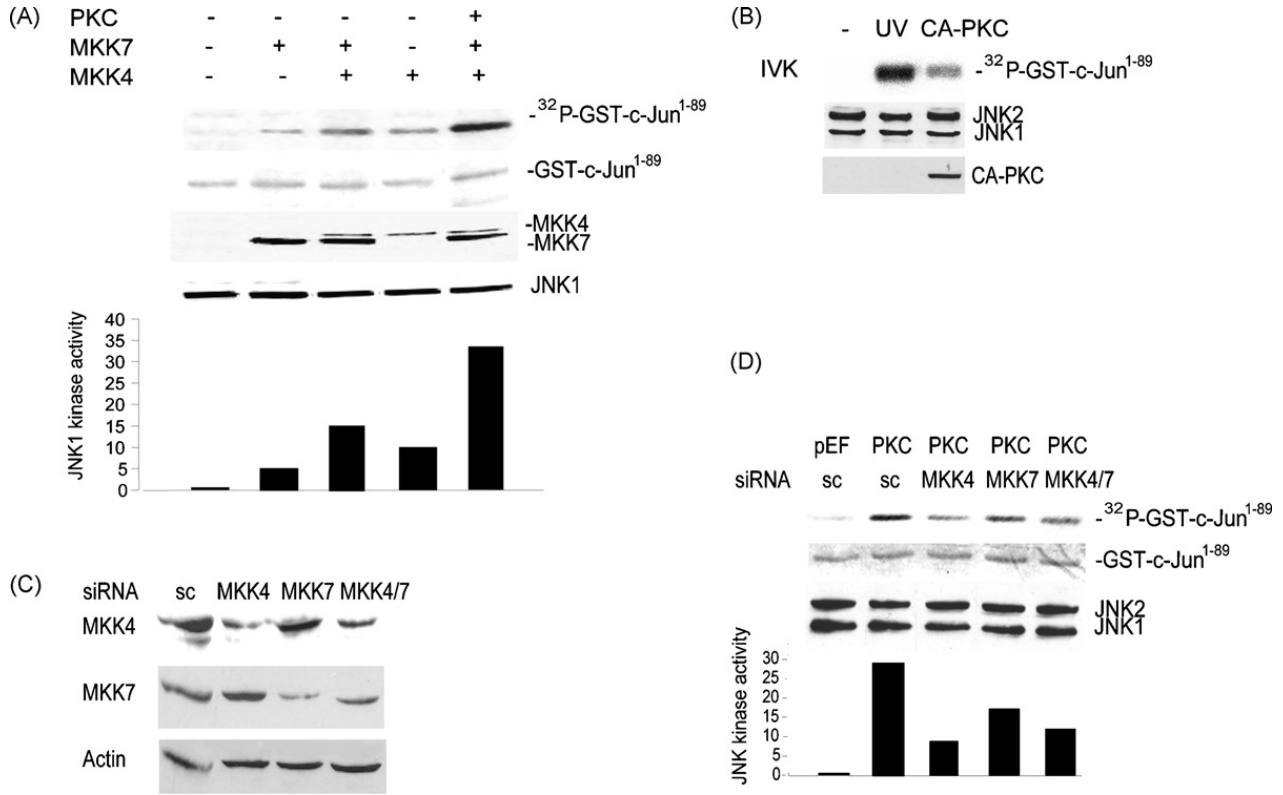


Fig. 4. The degree of JNK activation by MKK4-MKK7 is enhanced by PKC. (A) PKC augments JNK activation by MKK4/7. His-JNK1 (0.06 μM) was incubated with FLAG-MKK4 (0.03 μM), FLAG-MKK7 (0.12 μM) or both together in the presence of His-RACK1 (16 nM) and active PKC (18 nM). The degree of JNK activation was assessed in a kinase reaction using c-Jun as a substrate. (B) Activation of JNK by constitutive active PKC and UV-irradiation. HEK293T were transfected with CA-PKC (2 μg) or empty plasmid. Control cells were UV irradiated (40 J/m$^2$) 30 min before harvesting. Endogenous JNK was immunoprecipitated and its activity determined by an in vitro kinase reaction using c-Jun as a substrate. (C) Reduction in MKK4 and MKK7 expression levels by siRNA. Cells were transfected with CA-PKC (2 μg) and 100 μM of siRNA for MKK4 and/or MKK7 or scramble siRNA (sc). Extracts used in lane 4 were transfected with 50 μM of both siRNA for MKK4 and MKK7. (D) MKK4 and, to a lesser extent, MKK7 are required for PKC-dependent JNK activation. The activity of JNK in extracts described in C was assessed by an immunokinase reaction. Similar results were obtained in three separate experiments.

We next set out to assess whether, to achieve the maximal level of JNK activation, PKC phosphorylation must precede or follow MKK4/7. To this end, PKC and MKK4 or MKK7 were incubated with JNK in two sequential steps of 15 min each. Addition of PKC to a reaction containing JNK1 that had previously being incubated with MKK4 did not increase JNK activity over the levels induced by MKK4 alone (Fig. 3A). On the other hand, when MKK4 was added following incubation of JNK1 with PKC, the activity of JNK increased 2.5-fold (Fig. 3A). Similarly, addition of PKC prior to MKK7 resulted in higher JNK activity (Fig. 3B). These data suggest that to achieve a more efficient activation of JNK, phosphorylation by PKC should precede phosphorylation by MKK4 or MKK7. Since JNK is a stress-activated kinase and PKC is activated by various growth conditions, one would predict that to support maximal activation of JNK, PKC must be active prior to the presence of stimuli that activates MKK4/7 and consequently JNK. Alternatively, activation of PKC after MKK4/7 activation would enhance overall JNK activity by acting on remaining unphosphorylated JNK molecules in the cellular pool.

As dual-specificity kinases, MKK4 and MKK7 can phosphorylate both Tyr185 and Thr183. However, these two MKKs have different preferences for phosphoacceptor sites: Tyr185 for MKK4 and Thr183 for MKK7 (Fleming et al., 2000; Lin et al., 1995). We therefore assessed the role of PKC in JNK activation in the presence of both MKKs. As expected, MKK4 and MKK7 have an additive effect on JNK1 activation (Fig. 4A). When PKC was used in combination with MKK4 and MKK7, a further increase in JNK1 activity was seen (Fig. 4A). This result suggests that PKC cooperates with MKK4 and MKK7 to activate JNK1. We next elucidated the requirements of MKK4 and MKK7 for in vivo activation of JNK by PKC. Transfection of a constitutively active form of PKCβ (CA-PKC) efficiently activates JNK but to a lower degree than seen after UV-irradiation (Fig. 4B). To assess the role of MKK4 and MKK7 in PKC-dependent JNK activation we used specific siRNA that reduced MKK4 and MKK7 expression by approximately 70% (Fig. 4C). As shown in Fig. 4D, both MKK4 and MKK7 siRNAs reduced JNK activation induced by CA-PKC, although inhibition of MKK4 had a more pronounced effect on the degree of JNK activation. These results suggest that PKC enhanced JNK activation by both MKK4 and MKK7 although, both in vitro and in vivo, MKK4 appears to be the more potent kinase for JNK activation.

The data above reveal that PKC provides an additional layer in the regulation of JNK activity. To specifically investigate PKCs effects on the intensity and time course of JNK activity as well as its biological implications, we established cultures that overexpress JNK WT or JNK proteins mutated on the PKC or MKK4/7 sites. To this end we used SW1 melanoma cells in which endogenous levels of JNK1 and JNK2 were inhibited by a JNK1/2 siRNA (Fig. 5A). These cells were transfected with human wt JNK2, JNK-S129A (Fig. 5B), a mutant that cannot be phosphorylated by PKC (Fig. 3A) and JNK-APF [a JNK form in which alanines are substituted for the MKK4/7 phosphorylation sites (T183/Y185)]. Expression of these human JNK proteins was not affected by the JNK siRNA, which is directed against the mouse JNK sequence. As shown in Fig. 5C, the activity of JNK-WT was much higher than that of JNK-S129A. These data suggest that loss of PKC phosphorylation on JNK attenuates its degree of activation. The JNK-S129A mutant form of JNK, however, displays significantly higher levels of JNK activity than JNK-APF, which can no longer be phosphorylated by either MKK4 or MKK7 (Fig. 5C). To determine the contribution of classical PKC to JNK activation by UV, we used the cPKC inhibitor Go6976 (Lopez-Bergami et al., 2005). Consistent with the role of PKC in augmenting the degree of JNK activation, preincubation with the specific cPKC inhibitor Go6976 reduced the degree of JNK-WT activity following UV, but had little, if any, impact on the level of JNK-S129A activity (Fig. 5C).

The contribution of PKC to the overall level of JNK activity is expected to affect the biological activities regulated by JNK, including its regulation of programmed cell death. To directly assess this possibility, we measured the degree of apoptosis following UV-irradiation in SW1 cells expressing either JNK-WT or JNK-S129A. As shown in Fig. 5D, cells expressing JNK-S129A exhibited a lower degree of apoptosis following UV-irradiation compared with cells expressing JNK-WT. These data suggest that phosphorylation of JNK by PKC is an important contributor to the degree of JNK-induced apoptosis following UV-irradiation. Since the time course of JNK activation was shown to play an important part in apoptosis mediated by JNK (Ventura et al., 2006) we monitored possible effects of PKC on the kinetics of JNK activation following UV. Interestingly, Go6976 inhibited both the early (30 min) and late (8 h) phase of JNK activation following UV treatment (Fig. 5E). Altogether, our data illustrates PKC‘s role in augmenting the degree of JNK activity in concert with MKK4 and MKK7 under physiological conditions known to activate JNK.

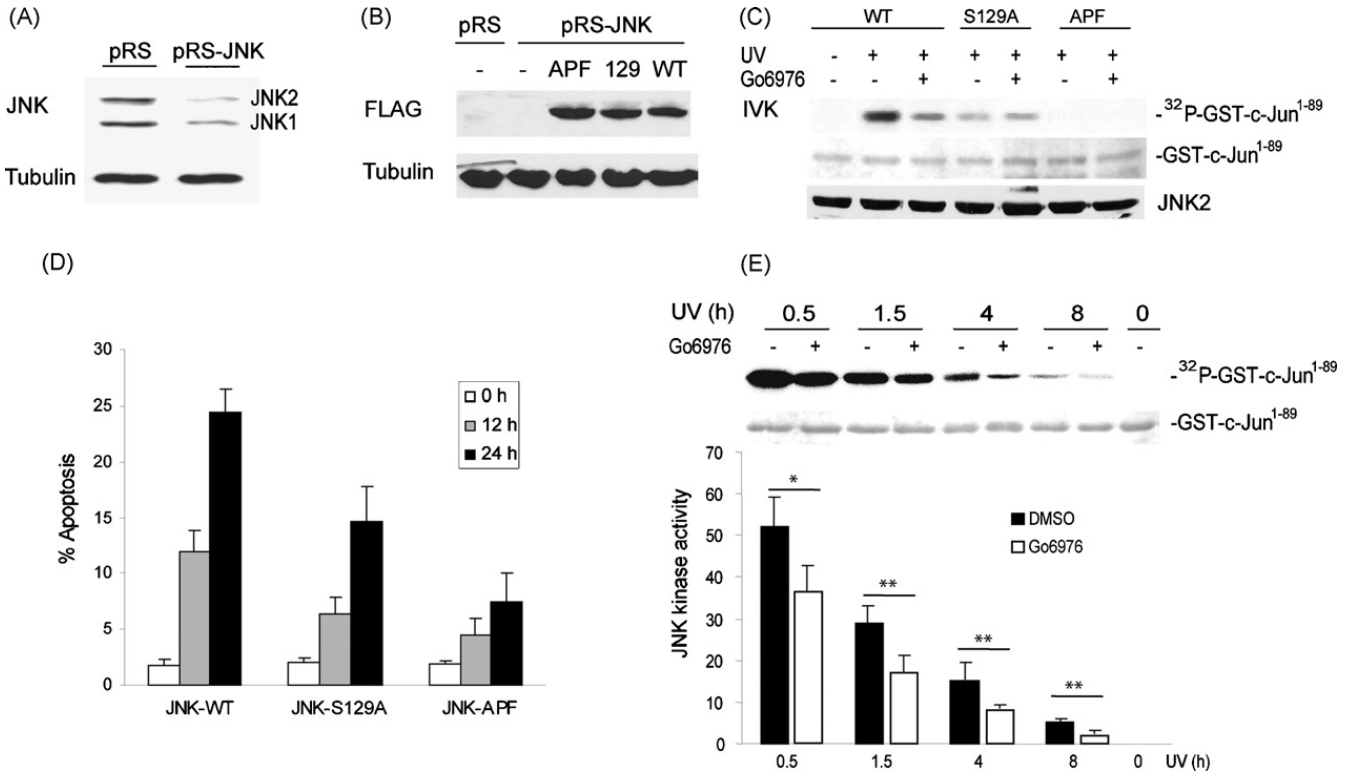


Fig. 5. Phosphorylation of JNK by PKC contributes to UV-induced apoptosis. Endogenous JNK levels were reduced by siRNA. SW1 cells were transfected with pRS or pRS-JNK (see Section 2) and stable lines resistant to puromycin were established. Expression of JNK was assessed by immunoblot. (B) Expression of JNK mutants in SW1 cells whose endogenous JNK levels were decreased by siRNA. SW1 cells described in A were transfected with FLAG-tagged JNK-WT, JNK-S129A and JNK-APF (an inactive form of JNK) and stable lines resistant to both puromycin and hygromycin were established. Expression of the exogenous proteins was assessed by immunoblot with FLAG antibody. (C) JNK-S129A is less active than JNK-WT. Cells described in B were treated with Go6976 for 30 min followed by UV treatment for an additional 30 min. Cells were lysed and exogenous JNK immunoprecipitated with FLAG antibodies. The activity of JNK in the immunoprecipitates was assessed by an immunokinase reaction using c-Jun as a substrate. (D) Cells expressing JNK-S129A are more resistant to UV-induced apoptosis. Cells described in B were UV-irradiated (40 J/m$^2$) and quantification of apoptotic cells was determined at the indicated time points. (E) The PKC inhibitor Go6976 inhibits both the early and late phases of JNK activation by UV. SW1 cells expressing both JNK siRNA and FLAG-JNK-WT were treated with Go6976 for 30 min followed by UV treatment (40 J/m$^2$). Protein extracts were obtained at the indicated time points following UV treatment. JNK activity was assessed by an immunokinase reaction using c-Jun as a substrate. Similar results were obtained in three separate experiments. *$p \leq 0.05$ and **$p \leq 0.01$.

## 4. Discussion

The mechanisms underlying the regulation of JNK activity are of great importance given the key role these kinases play in the regulation of cell survival or death in response to diverse extra-cellular stimuli. Along these lines, finding the contribution of MKK-independent signaling pathways to JNK activation and function allows better understanding of the network of signaling cascades engaged in the activation of these important protein kinases. Our recent studies documented the role of PKC and ERK in regulating JNK activity (Lopez-Bergami et al., 2005, 2007). Common to both signaling networks that activate JNK is the requirement for RACK1. While PKC activation of JNK requires RACK1 to allow direct phosphorylation of JNK (Lopez-Bergami et al., 2005), ERK contributes to the level of RACK1 via its impact on c-Jun expression (Lopez-Bergami et al., 2007).

In vitro activation of JNK by MKK4 and MKK7 has been well studied (Fleming et al., 2000; Lawler, Fleming, Goedert, & Cohen, 1998; Tournier et al., 2001). Following the finding that PKC contributes to JNK activation, we set to study the role of PKC on in vitro activation of JNK by MKK4 and MKK7. As in the previous publications (Fleming et al., 2000; Lawler et al., 1998; Tournier et al., 2001), the in vitro reactions were performed using purified kinases (JNKs and MKKs) and substrate (GST-c-Jun) either from bacterial cultures or immunoprecipitated from mammalian cells. In our experiments, however, we lowered the concentration of JNK more than 30 times (2 μM was used in articles by Lawler et al. (1998) and Fleming et al. (2000)) since in this condition we found a higher activation of JNK by PKC. Reducing JNK concentration also lowered the background of JNK2 activity. This background can be attributed in part to autophosphorylation of JNK2 (Pimienta et al. (2007)).

Our findings demonstrate the importance of PKC for JNK activation. Our data showed that phosphorylation by PKC enhances JNK activation by increasing MKK4/7 dependent phosphorylation. PKC phosphorylation of JNK did not affect MKK4-JNK association (Suppl. Fig. 4). Our findings also reveal that PKC phosphorylation of JNK must take place prior to its phosphorylation by MKK4 or MKK7 to augment the level of JNK activity. Although the phosphorylation by MKK4/7 does not require prior phosphorylation by PKC, as is the case with certain priming kinases, it is clearly augmenting JNK activity, once phosphorylated by both PKC and MKK4/7. That PKC must phosphorylate JNK prior to MKK4/7 to have an effect on the degree of JNK activity may be due to a conformational change. For example, MKK4/7 phosphorylation of JNK may reduce accessibility of S129 domain (which is close to the T loop) to PKC. (Cobb & Goldsmith, 1995; Hoofnagle, Resing, Goldsmith, & Ahn, 2001; Mittelstadt, Salvador, Fornace, & Ashwell, 2005). This finding is consistent with the notion that MKK4/7 rather than PKC are the immediate responders to stress or DNA damage. Thus, conditions that cause the activation of PKC may be part of the environment (cell type, growth conditions) rather than the actual response to stimuli, and as such, may result in basal phosphorylation of JNK on S129. Alternatively, PKC can be activated simultaneously or prior to MKKs by stimulus such as TPA, known to activate both pathways. This two-tier control mechanism is expected to be important in regard to the degree of JNK activity, and consequently, the degree of the biological output following its activation.

Our finding also implies that physiological conditions under which PKC is constitutively active would result in greater activation of JNK in response to stress, an example of such a situation is melanoma, where both ERK and PKC are constitutively upregulated, so that JNK and its overall output are enhanced as a result of the ERK-Jun-RACK1 link (Lopez-Bergami et al., 2007). Therefore, inhibition of PKC may better serve to attenuate, but not completely inhibit, the degree of JNK signaling. PKC thus emerges as a "fine tuner" of the JNK signaling pathway, an element that is often underappreciated in the "all or none" approach for therapeutic purposes. The particular contribution of different PKC isoforms to JNK activation can not be established from our in vitro experiments since we used a complex mixture of different PKCs.

Our data show that modulation by PKC affects both JNK1 and JNK2. Our findings also reveal that both in vitro and in vivo, MKK4 exhibits greater activity towards JNK, compared with MKK7. These findings are consistent with earlier reports regarding the degree of JNK activation by MKK4 versus MKK7 (Fleming et al., 2000; Lawler et al., 1998) although it is not clear whether these differences stem from the different phosphoacceptor site used by each of these kinases or the relative catalytic activity of the kinases themselves. Our data show that PKC cooperates with both MKK4 and MKK7 to activate JNK. However, the effect of PKC on JNK activity induced by MKK7 was twofold higher than on JNK activity induced by MKK4 (Fig. 2D and E). Further experiments are needed to clarify the nature of these differences.

Our data also reveal that inhibition of PKC phosphorylation of JNK affects both the early and the late phase of JNK activation, decreasing the pro-apoptotic response following UV radiation. Recently, Ventura et al. showed that the early transient phase of JNK activation (<1 h) can signal cell survival, whereas the later and more sustained phase of JNK activation (1–6 h) can mediate pro-apoptotic signaling (Ventura et al., 2006). Chang et al showed that sustained, but not transient, JNK activation promotes TNF-α dependent cell death via degradation of the caspase-8 inhibitor $cFLIP_L$ (Chang et al., 2006). These findings may indicate that by affecting JNK activity, PKC phosphorylation of JNK could contribute to both the anti-apoptotic (early phase) and the pro-apoptotic (late phase) response. However, the data obtained from cells expressing JNK-S129A seem to indicate that the pro-apoptotic response is affected to a greater degree by inhibition of PKCs effect on JNK. In all, the present study provides important insight into the mechanism underlying PKC phosphorylation of JNK as well as the implication of such phosphorylation for JNK activities.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2007.11.011.

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