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Cyclic AMP inhibits JNK activation by CREB-mediated induction of c-FLIP$_L$ and MKP-1, thereby antagonizing UV-induced apoptosis

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The cyclic AMP (cAMP) signaling pathway has been reported to either promote or suppress apoptosis, in a cell context-dependent manner. Our previous study has shown that cAMP, by protein kinase A (PKA)–cAMP response element-binding protein (CREB)–dynein light chain (DLC) pathway, negatively regulates mitogen-activated protein kinase p38 activation, thereby contributing to tumor necrosis factor (TNF)-α-induced apoptosis in certain types of cells. However, it remains largely unknown how cAMP suppresses apoptosis. Here we report that cAMP antagonized UV-induced apoptosis in Rat-1 and NIH 3T3 cells. Despite that cAMP significantly suppressed UV-induced p38 activation, inhibition of p38 activity showed no significant effect on UV-induced cell death in both cell lines. Further studies revealed that cAMP antagonized UV-induced apoptosis by inhibition of c-Jun N-terminal protein kinase (JNK) activation. The induction of the long form of cellular FLICE-inhibitory protein (c-FLIP$_L$) and mitogen-activated protein kinase phosphatase-1 (MKP-1), but not DLC and p21^{WAF1} by CREB was required for cAMP-mediated inhibition of JNK activation. The suppression by cAMP of UV-induced apoptosis was reversed by c-FLIP$_L$ small-interfering RNA (siRNA) or MKP-1 siRNA, which released the inhibition of JNK activation by cAMP. Thus, our results provide a molecular mechanism by which cAMP suppresses JNK activation and antagonizes apoptosis.
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JNK (c-Jun N-terminal protein kinase; also known as SAPK) is a member of the mitogen-activated protein kinase (MAPK) superfamily.[1] JNK has two ubiquitously expressed isoforms, JNK1 and JNK2, and a tissue-specific isoform JNK3, with different splicing forms (p54 and p46).[2,3] JNK is activated by sequential protein phosphorylation through a MAP kinase module, that is, MAPK kinase kinase (MAP3K) → MAPK kinase (MAP2K or MKK) → MAPK, in response to a variety of extracellular stimuli.[3] Two MAP2Ks, JNK-activating kinase 1 and 2 (JNKK1/MKK4 and JNKK2/MKK7), and several MAP3Ks are involved in JNK activation.[3] Once activated, JNK phosphorylates and regulates the activity of several transcription factors, such as c-Jun, ATF-2, Elk-1, p53, and c-Myc as well as nontranscription factors such as members of the Bcl-2 family proteins.[3,4] Overwhelming evidence shows that JNK has a central role in regulating many cellular activities from growth control to apoptosis.[2,3]

The second messenger cyclic AMP (cAMP) is produced from ATP by adenylyl cyclases (ACs) and can be degraded to 5′-AMP by phosphodiesterases.[5] AC is stimulated by a variety of extracellular stimuli, such as hormones, growth factors, and neurotransmitters through G-protein (Gs)-coupled membrane receptors.[5] In addition, AC can be activated by pharmacological agents, such as forskolin (FSK), which is a direct activator of AC,[6] isoproterenol, which is a synthetic agonist for the β-adrenergic family of receptors,[7] or cholera toxin (CTX), which causes constitutive activation of Gs by stimulating ADP-ribosylation of its α-subunit.[8] Protein kinase A (PKA) is the most important effector of cAMP action, although other cAMP-binding proteins, such as cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs), can mediate some of the biological functions of cAMP in a PKA-independent manner.[9,10] Active PKA can translocate into the nucleus to phosphorylate transcription factors, such as cAMP response element-binding protein (CREB) at Ser133.[11] The phosphorylation of CREB potentiates its transcription activity by recruitment of several transcription coactivators, such as CBP and p300,[11] thereby stimulating expression of target genes that are involved in many cellular activities, from proliferation to apoptosis.[12,13]

The cAMP signaling pathway has been reported to either promote or suppress apoptosis, in a cell context-dependent manner.[14–16] The underlying mechanisms remain elusive. Our recent work has revealed that the induction of dynein light chain (DLC) by CREB is required for cAMP-mediated inhibition of p38 activation.[17] The inhibition of p38 by cAMP

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Abbreviations: AC, adenylyl cyclases; ActD, actinomycin D; cAMP, cyclic AMP; c-FLIP$_L$, long form of cellular FLICE-inhibitory protein; CREB, cAMP response element-binding protein; DLC, dynein light chain; JNK, c-Jun N-terminal kinase; JNKK, JNK-activating kinase; MAPK, mitogen-activated protein kinase; MKP-1, mitogen-activated protein kinase phosphatase-1; PKA, protein kinase A; TNF-α, tumor necrosis factor-α
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leads to suppression of NF-κB activation and promotion of apoptosis in response to tumor necrosis factor-α (TNF-α).[17] There are three major groups of MAPKs in mammalian cells: extracellular signal-regulated kinase (ERK), JNK, and p38.[2] Numerous studies have shown that cAMP regulates ERK activity by various mechanisms.[18] Despite of the clarification of the mechanism by which cAMP inhibits p38 activation for apoptosis,[17] the same depth of knowledge about the mechanism by which cAMP regulates the activation of JNK is lacking. Furthermore, the mechanism by which cAMP suppresses apoptosis remains largely unknown. Here, we report that cAMP suppresses UV-induced apoptosis by inhibition of JNK activation. The induction of the long form of cellular FLICE-inhibitory protein (c-FLIP$_L$) and MAPK phosphatase-1 (MKP-1), but not DLC and p21^{WAF1} by CREB is required for cAMP-mediated inhibition of JNK activation.

Results

cAMP suppresses UV-induced apoptosis by a mechanism independent on p38 in Rat-1 and NIH 3T3 cells. Our previous study has shown that cAMP, by PKA–CREB–DLC pathway, negatively regulates p38 activation, thereby contributing to TNF-α-induced apoptosis in certain types of cells.[17] It is of interest to know whether cAMP plays a similar role in response to other types of death stimuli such as UV. For this purpose, Rat-1 and NIH 3T3 cells were pretreated with or without cAMP elevation agent FSK for 30 min, followed by stimulation with or without 20 J/m^2 UV and incubation for 16 h (20 J/m^2, 16 h). Hoechst staining showed that FSK significantly suppressed UV-induced cell death in both cell lines, even though FSK itself increased basal level of apoptosis (Figure 1a and b). Our previous study has shown that the suppression of p38 activation is the key mechanism by which cAMP promotes TNF-α-induced apoptosis. UV induces rapid and robust activation of p38. However, the exact roles of p38 in UV-induced apoptosis vary in a cell type-dependent manner.[19] It is unknown whether cAMP also inhibits UV-induced p38 activation and whether p38 plays an essential role in UV-induced apoptosis in Rat-1 and NIH 3T3 cells. Immunoblotting analysis revealed that UV-induced phosphorylation of p38 at Thr180 and Tyr182, which is required for p38 activation,[20] was significantly inhibited by FSK in Rat-1 and NIH 3T3 cells (Figure 1c and d). The role of p38 in UV-induced apoptosis in

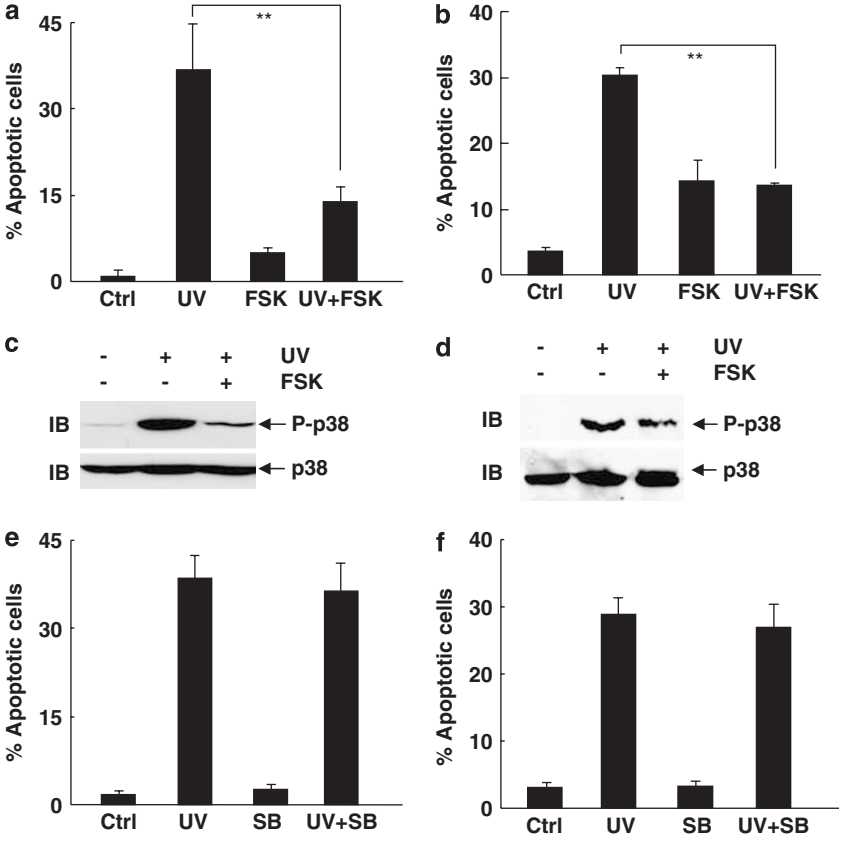

Figure 1 Cyclic AMP (cAMP) suppresses UV-induced apoptosis by a mechanism independent on p38 in Rat-1 and NIH 3T3 cells. (**a**, **b**) After pretreatment with or without forskolin (FSK, 20 μM) for 30 min, Rat-1 (**a**) and NIH 3T3 (**b**) cells were stimulated with or without 20 J/m^2 UV and incubated for 16 h (20 J/m^2, 16 h). Cell death was measured by Hoechst staining. Ctrl, control. Data are shown as mean $\pm$ standard deviations (S.D.); $n = 3$. $^{**}P < 0.01$ (Student's *t*-test). (**c**, **d**) After pretreatment with or without forskolin (FSK, 20 μM) for 30 min, Rat-1 (**c**) and NIH 3T3 (**d**) cells were stimulated with or without 20 J/m^2 UV and incubated for 20 min (20 J/m^2, 20 min). Phosphorylation and expression of p38 were analyzed by immunoblotting (IB) with phospho-p38 (P-p38) and p38 antibodies, respectively. (**e**, **f**) Rat-1 (**e**) and NIH 3T3 (**f**) cells were pretreated with or without SB203580 (SB, 10 μM) for 30 min, followed by stimulation with or without UV (20 J/m^2, 16 h). Cell death was measured by Hoechst staining

Rat-1 and NIH 3T3 cells was examined by using the selective p38 inhibitor, SB203580.[21] Rat-1 and NIH 3T3 cells were pretreated with 10 μM SB203580 for 30 min before exposure to UV radiation. This treatment protocol has been shown to result in the functional inhibition of p38 activity in Rat-1 and NIH 3T3 cells.[17] Pretreatment with SB203580 was found to have no effect on UV-induced apoptosis in both cell lines (Figure 1e and f). These findings suggest that p38 activation is not important in UV-induced apoptotic signaling in both Rat-1 and NIH 3T3 cells. Taken together, our data suggest that cAMP suppresses UV-induced apoptosis by a mechanism independent on p38 in Rat-1 and NIH 3T3 cells.

Elevation of cAMP inhibits JNK activity and phosphorylation, thereby antagonizing UV-induced apoptosis. There are three major groups of MAPKs in mammalian cells: ERK, JNK, and p38.[2] cAMP is a well-known regulator of MAP kinases in a cell type- and stimulus-dependent manner. Besides regulating p38 activation, elevation of cAMP can inhibit or activate ERK activity in a cell context-dependent manner.[18] cAMP also inhibits JNK activation with unknown mechanisms.[22,23] As it has been shown that UV-induced cell death is JNK-dependent,[24] it is important to investigate the regulation of JNK by cAMP. After pretreatment with or without cAMP elevation agent FSK for 30 min, Rat-1 cells were stimulated with or without 20 J/m^2 UV and incubated for 20 min (20 J/m^2, 20 min). Immune complex kinase assays showed that UV-stimulated JNK activity was significantly inhibited by FSK (Figure 2a). Immunoblotting analysis revealed that UV-induced phosphorylation of JNK at Thr183 and Tyr185, which is required for JNK activation,[3] was also significantly inhibited (Figure 2a). However, FSK showed no significant effect on UV-induced ERK phosphorylation (Figure 2a). UV-induced phosphorylation of JNK was also inhibited by FSK in NIH 3T3 cells (Figure 2b). The inhibition of JNK activity and

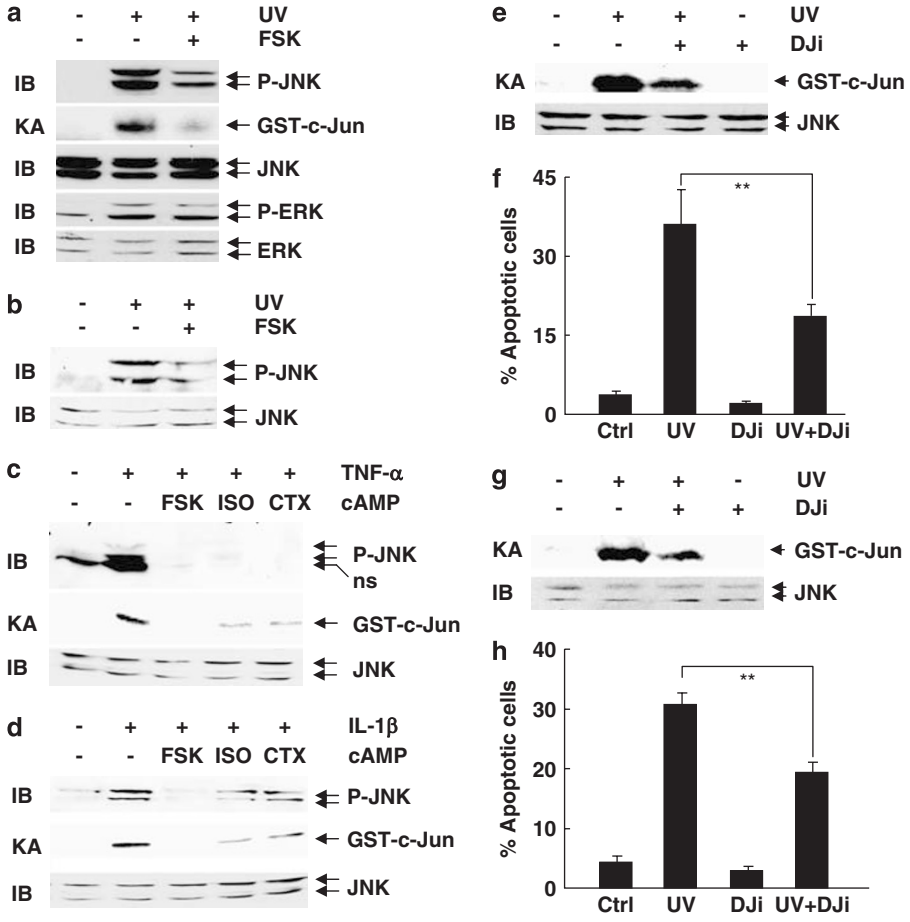

Figure 2 Elevation of cyclic AMP (cAMP) inhibits c-Jun N-terminal protein kinase (JNK) activity and phosphorylation, thereby antagonizing UV-induced apoptosis. (**a**, **b**) Rat-1 (**a**) and NIH 3T3 (**b**) cells were pretreated with or without forskolin (FSK, 20 μM) for 30 min, followed by stimulation with or without UV (20 J/m^2, 20 min). The activity of JNK was measured by immune complex kinase assays (KAs) with purified GST-c-Jun (2 μg) as the substrate. Phosphorylation and expression of JNK and extracellular signal-regulated kinase (ERK) were analyzed by immunoblotting with phospho-JNK (P-JNK), JNK, phospho-ERK (P-ERK), and ERK antibodies, respectively. (**c**, **d**) Rat-1 cells were pretreated with or without FSK (20 μM, 30 min), isoproterenol (ISO; 200 μM, 30 min), or cholera toxin (CTX; 100 ng/ml, 60 min), followed by stimulation with or without TNF-α (tumor necrosis factor-α) (**c**) or IL-1β (**d**) (5 ng/ml, 15 min each). The activity of JNK was measured by immune complex KAs. Phosphorylation and expression of JNK were analyzed by immunoblotting. (**e**–**h**) After pretreatment with or without D-JNKi1 (DJi, 10 μM) for 30 min, Rat-1 (**e**, **f**) and NIH 3T3 (**g**, **h**) cells were stimulated with or without 20 J/m^2 UV and incubated for 20 min (**e**, **g**) or for 16 h (**f**, **h**). The activity of JNK was measured by immune complex KAs with purified GST-c-Jun (2 μg) as the substrate (**e**, **g**). Cell death was measured by Hoechst staining with data shown as mean ± S.D.; $n = 3$. $^{**}P < 0.01$ (Student's *t*-test) (**f**, **h**)

phosphorylation by cAMP was not restricted to UV. Immunoblotting analysis and immune complex kinase assays revealed that TNF-α- or IL-1β-induced JNK activity and phosphorylation was also significantly inhibited by FSK and other cAMP elevation agents such as isoproterenol or CTX (Figure 2c and d). The role of JNK in UV-induced apoptosis in Rat-1 and NIH 3T3 cells was explored with the selective JNK inhibitor D-JNKi1 (DJi).[25] Pretreatment with 10 μM D-JNKi1 for 30 min significantly inhibited UV-stimulated JNK activity both in Rat-1 and NIH 3T3 cells (Figure 2e and g). Under the same conditions, D-JNKi1 significantly suppressed UV-induced apoptosis in these two cell lines (Figure 2f and h). Therefore, functional inhibition of JNK activity was enough to antagonize UV-induced apoptosis in Rat-1 and NIH 3T3 cells. Taken together, our data suggest that elevation of cAMP inhibits JNK activity and phosphorylation, thereby antagonizing UV-induced apoptosis.

The inhibition of JNK activation by cAMP depends on CREB-mediated transcription and involves upstream MAP2K. Our previous study has shown that the inhibition of p38 activation by cAMP requires *de novo* protein synthesis and depends on CREB-mediated transcription.[17] It is possible that cAMP inhibits JNK activation by the same mechanism. To test this scenario, Rat-1 cells were pretreated with or without FSK, followed by treatment with TNF-α or IL-1β in the presence or absence of the protein synthesis inhibitor emetine, or left untreated. The inhibition by FSK of TNF-α- or IL-1β-induced JNK phosphorylation was abolished by emetine (Figure 3a). TNF-α- or IL-1β-induced ERK phosphorylation was also inhibited by FSK. However, emetine had no reversible effects on ERK phosphorylation under the same conditions (Figure 3a). This suggests that *de novo* protein synthesis is required for the inhibition of JNK, but not ERK, by the cAMP pathway. In support of this notion, emetine or the RNA synthesis inhibitor actinomycin D also reversed the inhibition by FSK of UV-induced JNK phosphorylation (Figure 3b).

The cAMP pathway activates several transcription factors, including CREB, CREM, and ATF-1.[11–13] Among them, CREB is the major effector of the cAMP pathway.[11–13] CREB-specific inhibitor ACREB[26] has been shown to result in the blockade of FSK-induced CREB transcriptional activity and the reversal of the inhibition by FSK of TNF-α-induced p38 phosphorylation and activation in Rat-1 cells.[17] Under the same conditions, ACREB reversed the inhibition by FSK of TNF-α-induced JNK phosphorylation (Figure 3c). ACREB also reversed the inhibition by FSK of UV-induced JNK phosphorylation (Figure 3d). These data suggest that the inhibition of JNK by cAMP requires CREB, similar to the inhibition of p38 by cAMP.

The similarities of the mechanisms by which cAMP inhibits JNK and p38 activation promoted us to investigate whether cAMP also inhibited the kinase activity of the upstream MAP2K of JNK, like it does in the case of p38. Immune

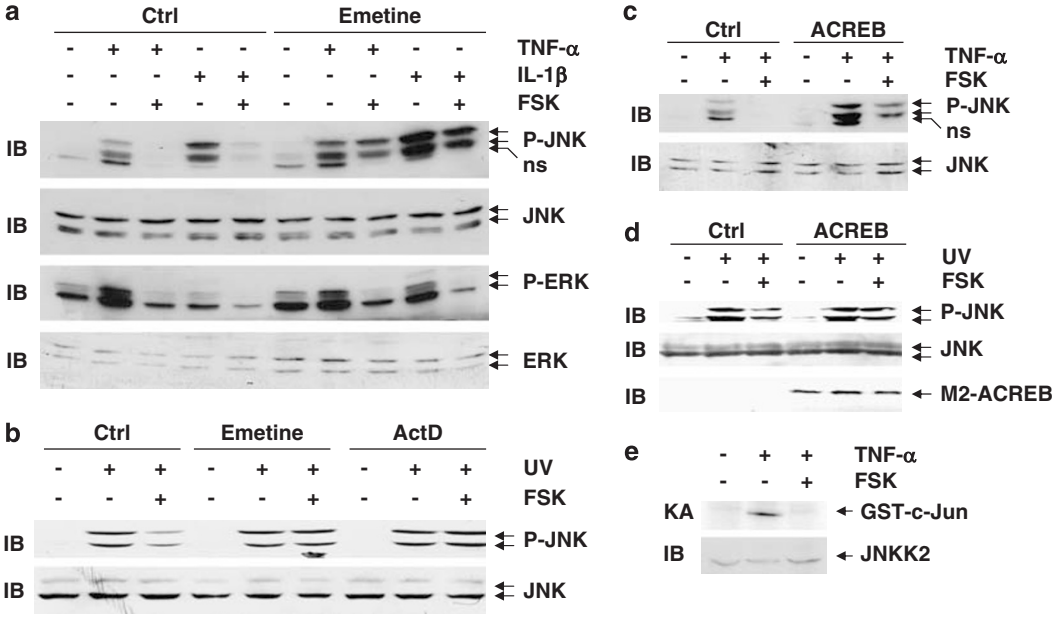

Figure 3 The inhibition of c-Jun N-terminal protein kinase (JNK) activation by cyclic AMP (cAMP) depends on cAMP response element-binding protein (CREB)-mediated transcription and involves upstream MAP2K. (**a**) Rat-1 cells were treated with or without emetine (1 μg/ml, 30 min) prior to forskolin (FSK) treatment (20 μM, 30 min), followed by stimulation with tumor necrosis factor-α (TNF-α) or IL-1β (5 ng/ml, 15 min), or left untreated. Phosphorylation and expression of JNK were determined. (**b**) Rat-1 cells were treated with or without emetine or actinomycin D (ActD; 1 μg/ml each, 30 min) prior to FSK treatment (20 μM, 30 min), followed by exposure to UV (20 J/m^2, 20 min), or left untreated. Phosphorylation and expression of JNK were determined. (**c**, **d**) Rat-1 cells were infected with adenovirus ACREB or the control adenovirus (multiplicity of infection = 300 each). After 48 h, cells were treated with FSK (20 μM, 30 min) prior to stimulation with TNF-α (5 ng/ml, 15 min) (**c**) or UV (20 J/m^2, 20 min) (**d**), or left untreated. Phosphorylation of JNK and expression of JNK and ACREB were determined. (**e**) Rat-1 cells were pretreated with or without FSK (20 μM, 30 min), followed by stimulation with or without TNF-α (5 ng/ml, 10 min). The activity of JNKK2 was measured by immune complex kinase assays. GST-JNK1 (0.01 μg) was used as substrate of active JNKK2, and GST-c-Jun (2–4 μg) was used as substrate of active GST-JNK1. The expression of JNKK2 was measured by immunoblotting

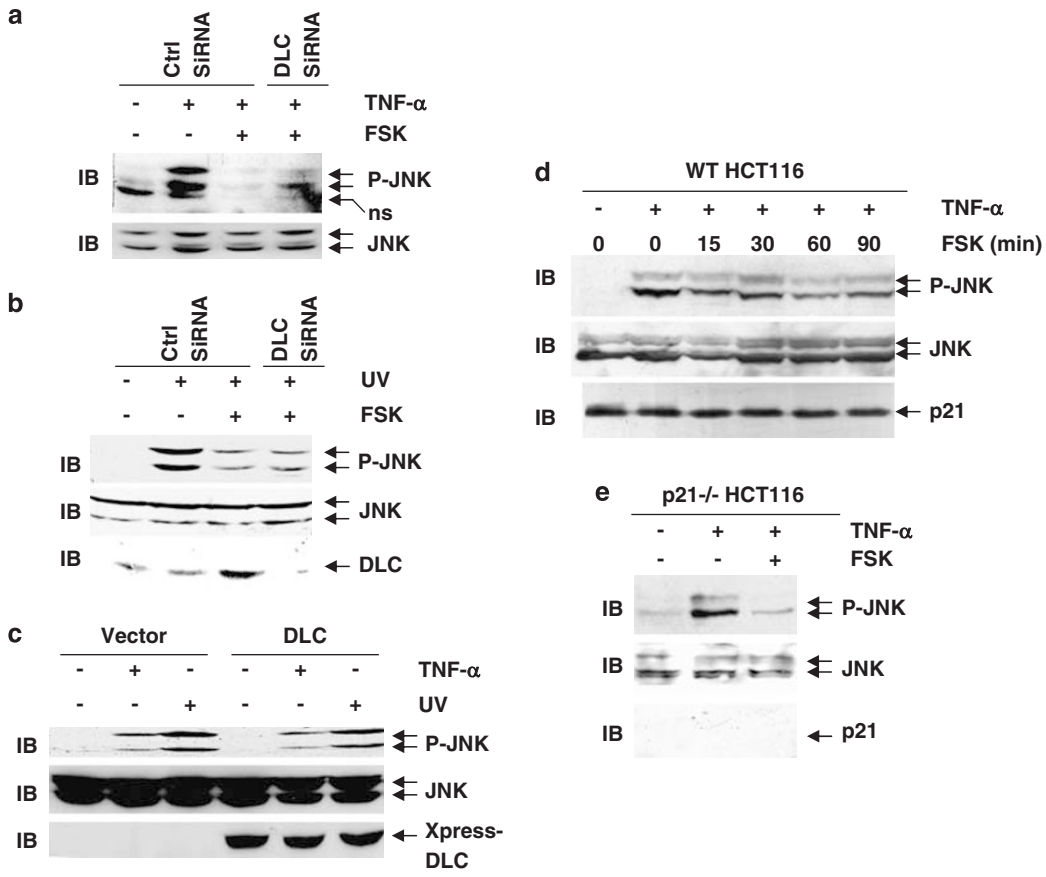

Figure 4 Dynein light chain (DLC) and p21^{WAF1} are not the major effectors of cyclic AMP (cAMP)-mediated inhibition of c-Jun N-terminal protein kinase (JNK) activation. (**a**, **b**) Rat-1 cells were transfected with DLC small-interfering RNA (siRNA) or the control scramble siRNA (100 nM each). After 48 h, cells were treated with or without forskolin (FSK, 20 μM, 30 min) prior to stimulation with tumor necrosis factor-α (TNF-α; 5 ng/ml, 15 min) (**a**) or UV (20 J/m^2, 20 min) (**b**) or left untreated. Phosphorylation of JNK and expression of JNK and DLC were determined. (**c**) Rat-1 cells were transfected with a mammalian expression vector encoding Xpress-DLC (4 μg). After 24 h, cells were stimulated with TNF-α (5 ng/ml, 15 min) or UV (20 J/m^2, 20 min) or left untreated. The phosphorylation of JNK and expression of Xpress-DLC and JNK were analyzed by immunoblotting. (**d**) HCT116/p21$+/+$ human colon carcinoma cells were pretreated with or without FSK (20 μM) for various times as indicated, followed by stimulation with or without TNF-α (5 ng/ml, 15 min). Phosphorylation of JNK and expression of JNK and p21^{WAF1} were analyzed by immunoblotting. WT, wild type. (**e**) HCT116/p21$-/-$ cells were pretreated with or without FSK (20 μM) for 60 min, followed by stimulation with or without TNF-α (5 ng/ml, 10 min). Phosphorylation of JNK and expression of JNK and p21^{WAF1} were analyzed by immunoblotting

complex kinase assays revealed that pretreatment of Rat-1 cells with FSK inhibited TNF-α-induced activation of JNKK2 (MKK7; Figure 3e). Taken together, our data suggest that the inhibition of JNK activation by cAMP depends on CREB-mediated transcription and involves upstream MAP2K, similar to the inhibition of p38 activation by cAMP.

DLC and p21^{WAF1} are not the major effectors of cAMP-mediated inhibition of JNK activation. Our previous study has shown that the induction of DLC by CREB is required for cAMP-mediated inhibition of p38 activation.[17] As the cAMP pathway suppresses JNK activation by a mechanism very similar to the inhibition of p38 activation, it is of interest to investigate whether DLC is required for the inhibition of JNK by cAMP. For this purpose, DLC small-interfering RNA (siRNA) was used to investigate the role of DLC in the inhibition of JNK by cAMP. Under the conditions that DLC siRNA significantly inhibited DLC expression and abolished the inhibition by FSK of TNF-α-induced p38 phosphorylation,[17] DLC siRNA showed only marginal effect on the inhibition by FSK of TNF-α-induced JNK phosphorylation (Figure 4a). Furthermore, DLC siRNA had no significant effect on the inhibition by FSK of UV-induced JNK phosphorylation (Figure 4b). Consistent with this, ectopic expression of DLC in Rat-1 cells had no significant effect on TNF-α or UV-stimulated JNK phosphorylation (Figure 4c). Therefore, DLC is not the major effector of cAMP-mediated inhibition of JNK activation.

Because p21^{WAF1} has been shown to inhibit JNK activation in various cells and has been reported to exert a protective effect against radiations and various chemical apoptosis inducers,[27,28] we next asked whether p21^{WAF1} might be involved in the inhibition of JNK activation by cAMP. For this purpose, we used wild-type HCT116/p21$+/+$ human colon carcinoma cells and HCT116/p21$-/-$ cells in which both *p21WAF1* alleles have been deleted by homologous recombination.[28] Immunoblotting analysis revealed that TNF-α-induced JNK phosphorylation was significantly inhibited by FSK in wild-type HCT116/p21$+/+$ human colon carcinoma cells. The maximal inhibition occurred upon pretreatment with

FSK for 60 min (Figure 4d). Similarly, FSK pretreatment for 60 min led to significant inhibition of TNF-α-induced JNK phosphorylation in HCT116/p21$-/-$ cells (Figure 4e). FSK pretreatment for 60 min also led to weak but similar inhibition of UV-induced JNK phosphorylation in both cell lines (data not shown). Furthermore, FSK showed no effect on the expression of $p21^{WAF1}$ (Figure 4d). Taken together, these data suggest that $p21^{WAF1}$ is not the major effector of cAMP-mediated inhibition of JNK activation.

cAMP by CREB induces expression of c-FLIP$_L$ and MKP-1, which is well correlated with the inhibition of JNK activation by cAMP. Among the known JNK inhibitors, MKP-1 has been shown to be upregulated by cAMP,[29,30] whereas recent studies revealed that there is CREB site in the promoter region of c-FLIP$_L$ and loss of CREB function correlated with decreased expression of c-FLIP$_L$.[31,32] These observations led us to test whether these two inhibitors are involved in the inhibition of JNK by cAMP. Immunoblotting analysis revealed that FSK induced expression of both c-FLIP$_L$ and MKP-1 in Rat-1 and NIH 3T3 cells (Figure 5a and b). FSK-induced expression of c-FLIP$_L$ and MKP-1 in Rat-1 cells occurred as soon as 15 min after the treatment, but became evident 30 min after the treatment and decreased 120 min after the treatment (Figure 5c). The kinetics of FSK-induced expression of c-FLIP$_L$ and MKP-1 was correlated with its effects on phosphorylation of CREB at Ser133 (Figure 5c), and inhibition of JNK phosphorylation (Figure 5d). CREB-specific inhibitor ACREB was used to determine whether c-FLIP$_L$ and MKP-1 are upregulated by FSK by CREB. Immunoblotting analysis revealed that FSK-induced expression of c-FLIP$_L$ and MKP-1 was inhibited by ACREB (Figure 5e). Taken together, our data suggest that cAMP by CREB induces expression of c-FLIP$_L$ and MKP-1, which is well correlated with the inhibition of JNK activation by cAMP.

c-FLIP$_L$ siRNA or MKP-1 siRNA released the inhibition of JNK activation by cAMP, and reversed the suppression of UV-induced apoptosis by cAMP. If cAMP inhibits JNK activation by induction of c-FLIP$_L$ and MKP-1, inhibition of c-FLIP$_L$ or MKP-1 expression should abrogate the inhibition by cAMP of JNK activation. To test this hypothesis, Rat-1 cells were transfected with c-FLIP$_L$ siRNA, or MKP-1 siRNA, or negative control siRNA, pretreated with or without FSK, and then stimulated with UV (20 J/m^2, 20 min) or left alone. Immunoblotting analysis revealed that FSK induced expression of c-FLIP$_L$ and MKP-1 and inhibited UV-induced JNK and p38 activation (Figure 6a). Transfection of the cells with c-FLIP$_L$ siRNA, which significantly inhibited c-FLIP$_L$

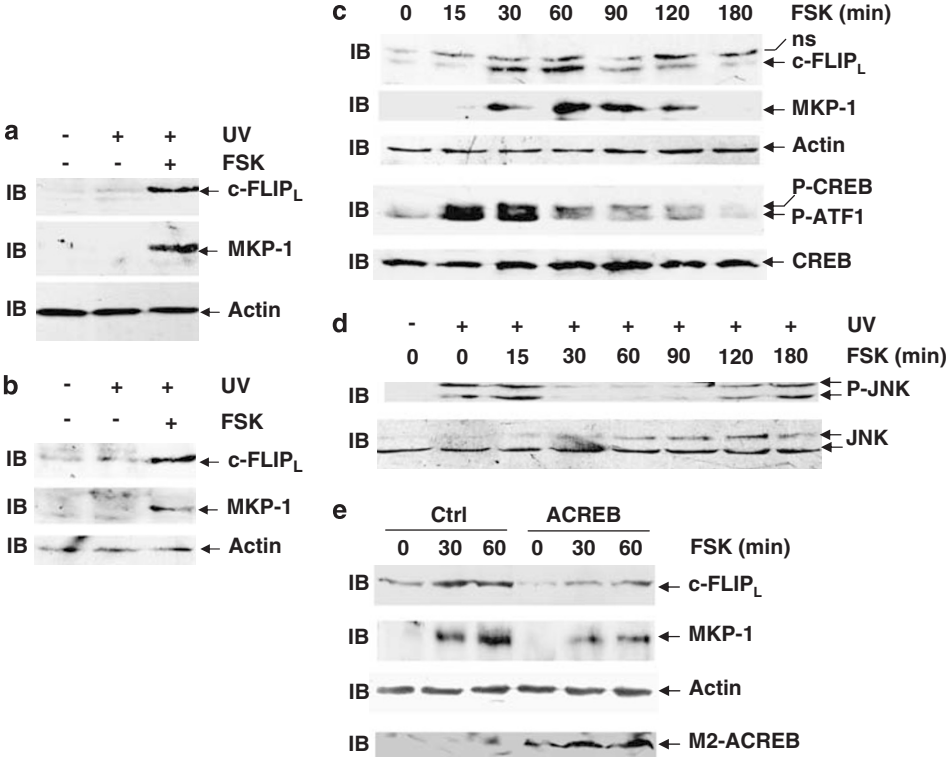

Figure 5 Cyclic AMP (cAMP) by cAMP response element-binding protein (CREB) induces expression of the long form of cellular FLICE-inhibitory protein (c-FLIP$_L$) and mitogen-activated protein kinase phosphatase-1 (MKP-1), which is well correlated with the inhibition of c-Jun N-terminal protein kinase (JNK) activation by cAMP. (**a**, **b**) Rat-1 (**a**) and NIH 3T3 (**b**) cells were pretreated with or without forskolin (FSK, 20 μM) for 30 min, followed by stimulation with or without UV (20 J/m^2, 20 min). Expression of c-FLIP$_L$, MKP-1 and actin was analyzed by immunoblotting. (**c**) Rat-1 cells were treated with FSK (20 μM) for various times as indicated. Expression of c-FLIP$_L$, MKP-1, and actin was analyzed by immunoblotting. Phosphorylation and expression of CREB were also determined by immunoblotting. Please Note that phospho-CREB (P-CREB) antibody can detect phospho-ATF1 (P-ATF1). (**d**) Rat-1 cells were pretreated with FSK (20 μM) for various times as indicated, followed by stimulation with or without UV (20 J/m^2, 20 min). Phosphorylation and expression of JNK were analyzed by immunoblotting. (**e**) Rat-1 cells were treated as described in Figure 3c. Cells were treated with FSK (20 μM) for various times as indicated. Expression of c-FLIP$_L$, MKP-1, actin and ACREB was analyzed by immunoblotting

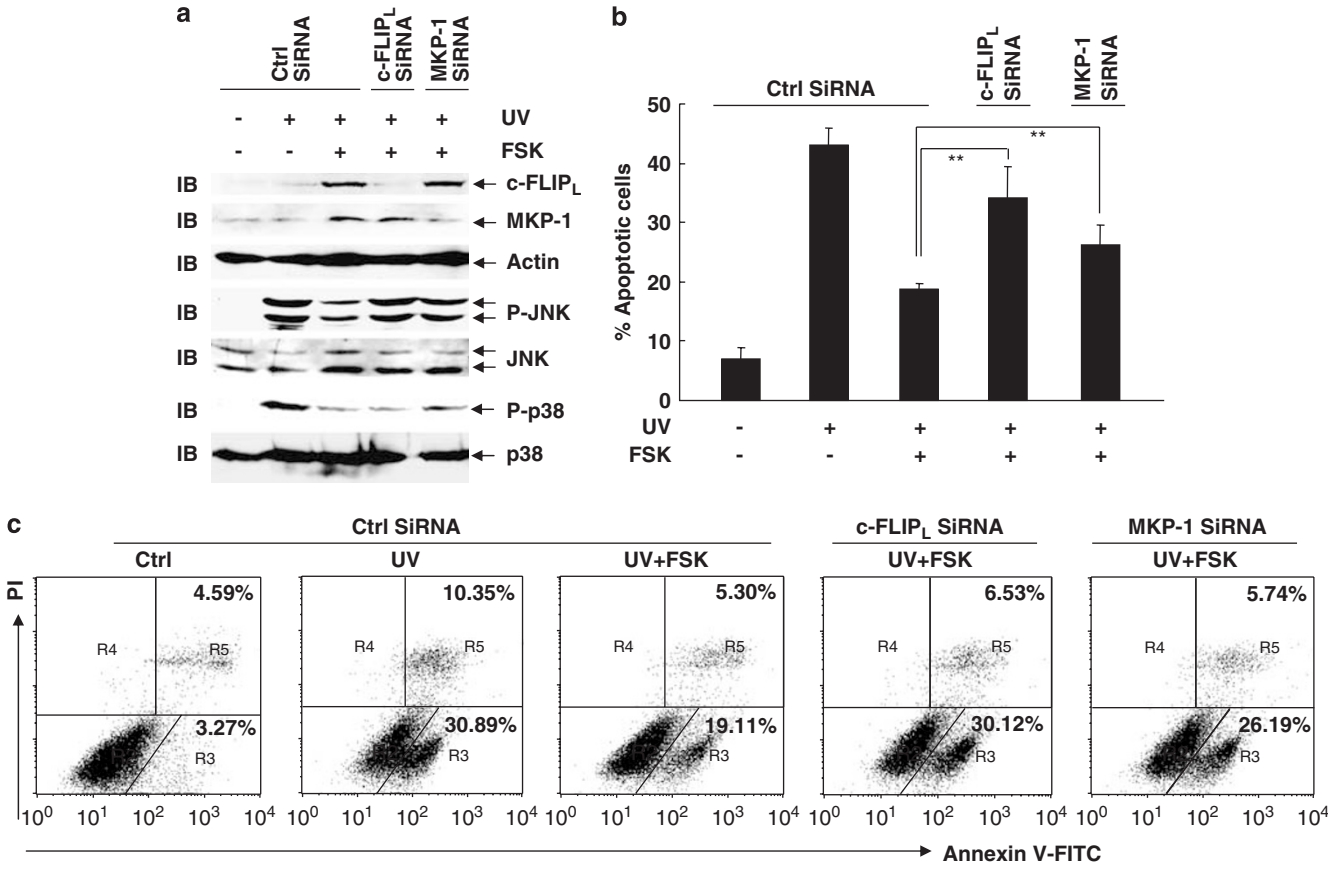

Figure 6 Long form of cellular FLICE-inhibitory protein (c-FLIP$_L$) small-interfering RNA (siRNA) or mitogen-activated protein kinase phosphatase-1 (MKP-1) siRNA released the inhibition of c-Jun N-terminal protein kinase (JNK) activation by cyclic AMP (cAMP), and reversed the suppression by cAMP of UV-induced apoptosis. (**a**) Rat-1 cells were transfected with c-FLIP$_L$ siRNA or MKP-1 siRNA or the negative control siRNA (100 nM each). After 48 h, cells were treated with or without forskolin (FSK, 20 μM, 30 min), followed by stimulation with or without UV (20 J/m^2, 20 min). Phosphorylation of JNK and p38 and expression of c-FLIP$_L$, MKP-1, actin, JNK, and p38 were determined. (**b**, **c**) Rat-1 cells were treated as described in Figure 6a. After FSK pretreatment, Rat-1 cells were stimulated with UV (20 J/m^2, 16 h). Cell death was measured by Hoechst staining with data shown as mean $\pm$ S.D.; $n=3$. $^{**}P<0.01$ (Student's *t*-test) (**b**) or by Annexin V/ PI staining (**c**)

expression, but not MKP-1 expression, abolished the inhibition by FSK of UV-induced JNK activation, but not p38 activation (Figure 6a). By contrast, transfection of the cells with MKP-1 siRNA, which significantly abolished MKP-1 expression, but not c-FLIP$_L$ expression, partially reversed the inhibition by FSK of UV-induced JNK activation, as well as p38 activation (Figure 6a).

Our previous data have shown that elevation of cAMP inhibits JNK activation, thereby antagonizing UV-induced apoptosis (Figure 2). As c-FLIP$_L$ siRNA or MKP-1 siRNA released the inhibition of JNK activation by cAMP, they should also reverse the suppression by cAMP of UV-induced apoptosis. Indeed, Hoechst staining revealed the inhibition by FSK of UV-induced apoptosis was significantly reversed by c-FLIP$_L$ siRNA or MKP-1 siRNA (Figure 6b). Annexin V/PI staining showed similar results (Figure 6c).

Discussion

The MAPK JNK regulates many cellular events, from cell-cycle progression to apoptosis, and the activity of JNK itself is tightly controlled by other intracellular signaling pathways, such as NF-κB. It has been shown that NF-κB prevents prolonged JNK activation in response to TNF-α, which otherwise contributes to TNF-α killing.[3,33,34] UV induces rapid and prolonged JNK activation, which is essential for UV-killing.[24] NF-κB is required for UV-induced JNK activation by induction of PKCδ, which provides a mechanism by which NF-κB promotes UV-induced apoptosis.[35] Besides NF-κB, the cAMP pathway has been reported to inhibit JNK activation with unknown mechanisms.[22,23] Here, we show the induction of c-FLIP$_L$ and MKP-1, but not DLC and p21^{WAF1} by CREB is required for cAMP-mediated inhibition of JNK activation. Our results revealed the molecular mechanism underlying the cross talk between the cAMP and JNK pathways.

MKP-1 has been shown to be upregulated by cAMP,[29,30] whereas recent studies revealed that there is CREB site in the promoter region of c-FLIP$_L$ and loss of CREB function correlated with decreased expression of c-FLIP$_L$.[31,32] Here, our results reveal that, at least in fibroblasts, cAMP induces the expression of both MKP-1 and c-FLIP$_L$ (Figure 5a, b, and c) and CREB is essential in the induction of c-FLIP$_L$ and MKP-1 (Figure 5e).

c-FLIP$_L$ and MKP-1 inhibit JNK activation by distinct mechanisms. MKP-1 directly dephosphorylates MAPK family members ERK, JNK, and p38. However, the ability of MKP-1

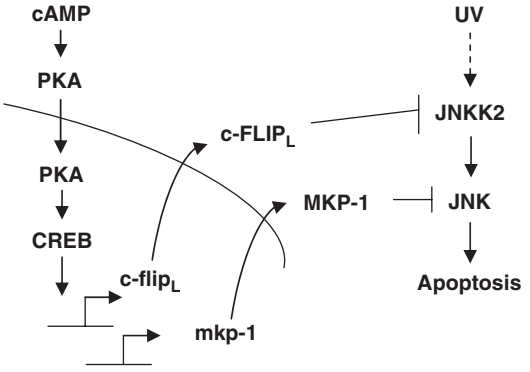

Figure 7 A schematic presentation of the molecular mechanism by which cyclic AMP (cAMP) inhibits c-Jun N-terminal protein kinase (JNK) activation and suppresses apoptosis

to dephosphorylate each one of these kinases varies depending on the cell system and its environmental conditions.[36,37] Conditional expression of MKP-1 was found to abolish UV-induced JNK activity, and inhibit UV-induced apoptosis.[38] In this report, we found that knockdown of MKP-1 expression led to partial reversal of the inhibition by cAMP of UV-induced apoptosis as well as JNK activation (Figure 6). Therefore, MKP-1 is indeed involved in the cross talk between the cAMP and JNK pathways (Figure 7).

The induction of c-FLIP$_L$ might cooperate with MKP-1 to inhibit JNK activation. c-FLIP$_L$ has been shown to bind directly to FADD and caspase-8 by DEDs, thereby inhibiting death receptor-mediated apoptosis.[39] In addition, a recent study has revealed that c-FLIP$_L$ directly interacts with JNKK2 (MKK7) in a TNF-α-dependent manner and downregulates JNK activation.[40] Here, we show that cAMP-induced c-FLIP$_L$ expression inhibits UV-induced JNK activation because c-FLIP$_L$ siRNA significantly released the inhibition by cAMP of UV-induced JNK activation as well as apoptosis (Figure 7). It is possible that both TNF-α and cAMP induce some component required for the efficient interaction between c-FLIP$_L$ and JNKK2. Further studies are required to clarify this issue.

It is interesting that cAMP inhibits the activation of p38 and JNK by overlapping but by different mechanisms. Our previous study revealed that the induction of DLC by CREB is required for cAMP-mediated inhibition of p38 activation. In this report, our data show that the inhibition of JNK activation by cAMP also depends on CREB-mediated transcription and involves upstream MAP2K. Further analysis suggests the induction of c-FLIP$_L$ and MKP-1, but not DLC and p21^{WAF1} by CREB is required for cAMP-mediated inhibition of JNK activation. The induction of MKP-1 also contributes to the inhibition by cAMP of p38 activation because MKP-1 siRNA, but not c-FLIP$_L$ siRNA, partially released the inhibition by cAMP of p38 activation (Figure 6a).

The exact role of JNK in apoptosis is really cell context-dependent.[3] UV killing is JNK-dependent.[24] cAMP inhibits JNK, thereby suppresses UV killing. Knockdown of the induction of c-FLIP$_L$ and MKP-1 by cAMP released the inhibition by cAMP of UV-induced JNK activation, and consequently reversed the antiapoptotic role of cAMP in UV killing (Figure 6). The antiapoptotic role of c-FLIP$_L$ might also come from its ability to bind directly to FADD and caspase-8 by DEDs.[39] cAMP also regulates other pathways such as ERK and p38.[17,18] The interplay or the balance of these pathways determines the outcome. Our previous study has shown that cAMP, by CREB-induced DLC expression, negatively regulates p38 activation, thereby contributing to TNF-α-induced apoptosis in certain types of cells.[17] So the cAMP pathway is sometimes antiapoptotic, sometimes proapoptotic, depending on the cell context.

Materials and Methods

Cell culture. Rat-1, NIH 3T3, p21$-/-$ HCT, and wild-type control cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Reagents. Antibody against Flag (M2) tag, FSK, isoproterenol, CTX, Hoechst 33258, emetine, and actinomycin D were from Sigma. Antibodies against phospho-JNK, phospho-ERK, ERK, phospho-p38, p38, phospho-CREB, and CREB were from Cell Signaling. Antibodies against Xpress tag, DLC, actin, JNKK2, c-FLIP$_L$, MKP-1, and p21 were from Santa Cruz. JNK antibodies (antibody 666 and antibody 333) and Annexin V kit were from BD Company. Mouse IL-1β and TNF-α were purchased from R&D Systems. SB203580 was from Calbiochem. D-JNKi1 was purchased from BioMol. [γ-^{32}P]ATP (3000 mCi/nmol) was from Dupont NEN.

RNA interference and adenoviruses. siRNAs that target rat DLC, rat c-FLIP$_L$, and rat MKP-1 mRNAs were designed based on nucleotides 532–550 (DLC), 904–922 (c-FLIP$_L$), and 245–263 (MKP-1) relative to the translation start sites, respectively, and purchased from Dharmacon. Cells were transfected with siRNAs using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. Recombinant adenovirus containing *ACREB* (provided by Chen-Ming Fan, Carnegie Institution of Washington) was constructed by subcloning ACREB cDNA into pTRACK CMV and then inserting it into pAdeasy-1 by homologous recombination.[26] The viral preparation and purification was carried out as described previously.[17] Cells were infected with adenovirus at a final concentration of 3×10^{10} PFU/ml (multiplicity of infection $=$ 300).

Immune complex kinase assays and immunoblotting analysis. Immune complex kinase assays were performed and quantitated as described previously.[4] Briefly, active JNKK2 or JNK1 was immunoprecipitated using anti-JNKK2 antibody or anti-JNK1 antibody (antibody 333), respectively. Kinase assays were carried out for 60 min at 30°C in 20 mmol/l HEPES (pH 7.6), 20 mmol/l $MgCl_2$, 1 mmol/l DTT, 10 μmol/l nonradioactive ATP, and 10 μCi [γ-^{32}P]ATP. For measuring JNK1 activity, GST-c-Jun (2–4 μg) was used as substrate. For measuring JNKK2 activity, GST-JNK1 (0.01 μg) was used as substrate, and GST-c-Jun (2–4 μg) was used as substrate of active GST-JNK1. Immunoblotting analysis was done as described previously.[4]

Apoptosis assays. Cells were stained with Hoechst (H33258), and nuclear condensation and DNA fragmentation were visualized by fluorescence microscopy, as described previously.[4] Annexin V/PI staining was performed according to the manufacturer's protocol with Annexin V kit.

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