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The conditional kinase ΔMEKK1:ER* selectively activates the JNK pathway and protects against serum withdrawal-induced cell death

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Abstract

The conditional protein kinase ΔMEKK3:ER* allows activation of the mitogen-activated and stress-activated protein kinases (MAPKs and SAPKs) without imposing a primary cellular stress or damage. Such separation of stress from stress-induced signalling is particularly important in the analysis of apoptosis. Activation of ΔMEKK3:ER* in cycling CCl39 cells caused a rapid stimulation of the ERK1/2, JNK and p38 pathways but resulted in a slow, delayed apoptotic response. Paradoxically, activation of the same pathways inhibited the rapid expression of Bim_{EL} and apoptosis following withdrawal of serum. Inhibition of the ERK1/2 pathway prevented the down-regulation of Bim_{EL} but caused only a partial reversion of the cyto-protective effect of ΔMEKK3:ER*. In contrast, inhibition of p38 had no effect, raising the possibility that activation of JNK might also exert a protective effect. To test this we used CCl39 cells expressing ΔMEKK1:ER* which activates JNK but not ERK1/2, p38, PKB or IκB kinase. Activation of ΔMEKK1:ER* inhibited serum withdrawal-induced conformational changes in Bax and apoptosis. These results suggest that in the absence of any overt cellular damage or chemical stress activation of JNK can act independently of the ERK1/2 or PKB pathways to inhibit serum withdrawal-induced cell death.

Keywords: Apoptosis; Bim; ERK; JNK; MEKK1; MEKK3

1. Introduction

The Ras-regulated Raf→MEK1/2→ERK1/2 cascade is activated by growth factors and promotes cell cycle re-entry [1,2] but can also promote cell survival [3]. Activation of ERK1/2 inhibits apoptosis induced by ionizing radiation [4], anchorage removal [5] or the withdrawal of survival factors [5,6]. This may reflect ERK1/2-dependent transcriptional up-regulation of the pro-survival Bcl-2 proteins [8,9]. However, ERK1/2 can also target specific 'BH3-only' proteins that are involved in initiating the cell death pathway. For example, during withdrawal of survival factors Bad is de-phosphorylated and activated, whereas activation of the ERK1/2 pathway can promote the $p90^{RSK}$-dependent phosphorylation and sequestration of Bad [10]. Similarly, activation of the ERK1/2 pathway can block the de novo expression of Bim, which is induced following withdrawal of serum [7,11].

Abbreviations: Bad, Bcl-2 antagonist of cell death; BH, Bcl-2 homology domain; Bim, Bcl-2 interacting modulator; Bim_{EL}, Bim extra long; ERK, Extracellular signal-regulated kinase; FBS, Fetal bovine serum; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; MEK, MAPK or ERK Kinase; MEKK, MEK Kinase; NGF, Nerve growth factor; PI3K, Phosphatidylinositol 3′-kinase; PKB, Protein kinase B; $p90^{RSK}$/RSK, Ribosomal protein S6; kinase; SAPK, Stress-activated protein kinase; SB203580, [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole]; U0126, 1,4-diamino-2,3-dicyano-1,4*bis*(2-aminophenylthio)butadiene; 4-HT, 4-hydroxytamoxifen.

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In contrast to the ERK1/2 pathway, activation of the c-Jun N-terminal kinase (JNK) pathway is generally considered to be a pro-apoptotic signal. For example, JNK is activated following withdrawal of NGF from primary sympathetic neurons [12] where it promotes the expression of Bim [13,14], FasL [15] and cell death. JNK-dependent activation of c-Jun is also required for expression of FasL and cell death following exposure to DNA alkylating agents [16]. Most importantly, *Jnk1-/-Jnk2-/-*MEFs, which lack JNK activity, show greatly reduced apoptosis in response to UV radiation and alkylating agents [17].

However, JNK is not simply a pro-apoptotic signal and many stimuli that activate JNK fail to elicit cell death [18]. Indeed, several studies have shown that activation of JNK can exert a cyto-protective effect under certain conditions. For example, following stimulation with TNFα, activation of JNK does not correlate with cell death [19] and may even act to promote cell survival [20,21]. JNK promotes cell survival in transformed B lymphoblasts [22] and provides an adhesion-dependent survival signal in a pathway downstream of fibronectin and focal adhesion kinase [23]. Finally, *Jnk1-/-Jnk2-/-*mutant embryos display increased apoptosis in the developing forebrain [24,25] and disruption of *MKK4* (a JNK activator) [26] or *c-Jun* (a JNK target) [27] causes embryonic lethality associated with increased apoptosis in the liver suggesting that JNK acts to coordinate development with cell survival.

In seeking to define the role of JNK in cell death it is important to be able to separate JNK activation from the cellular damage that often accompanies it. To this end we have developed two conditional kinases that allow activation of JNK, either alone (ΔMEKK1:ER*) or in combination with ERK and p38 (ΔMEKK3:ER*), without causing any cellular damage or stress [28,29]. Here we show that whilst activation of ΔMEKK3:ER* causes a strong apoptotic response in cycling cells, it actually protects the same cells from serum withdrawal-induced cell death. This protective effect correlates in part with the ERK1/2-dependent down-regulation of Bim. However, when ERK1/2 and p38 are inhibited we still observe a significant protective effect. Using ΔMEKK1:ER we now show that selective activation of the JNK pathway can also protect cells from serum withdrawal-induced cell death.

2. Materials and methods

2.1. Materials

Cell culture reagents were purchased from Gibco Life Technologies. Superfect transfection reagent was supplied by Qiagen. CPP32 substrate (Ac-DEVD-AMC) was from Calbiochem. U0126 was purchased from Promega. [γ-^{32}P] ATP was routinely purchased from Amersham. The following antibodies were used throughout this study: ERK1, JNK1 and p38α were prepared in house; phospho-ERK1/2, total ERK1/2, phospho (Ser63) c-Jun, phospho (Ser473) PKB, total PKB and cleaved caspase 3 (17kD fragment) were from Cell Signalling Technology/NEB; Bcl-2 and Bad were from Santa Cruz Biotechnology; Bax (N-20) was from Calbiochem and Bim from Chemicon. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Unless otherwise stated in the text, all other chemicals were purchased from Sigma and were of the highest grade available.

2.2. Cells and cell culture

CM 3 cells are CCl39 fibroblasts expressing ΔMEKK3:ER* and have been described in detail previously [28]. CM 1 cells are CCl39 fibroblasts expressing ΔMEKK1:ER*. The selective activation of the JNK pathway by ΔMEKK1:ER* in these cells has been described in three studies [7,29,34]. CM1 and CM3 cells were maintained in Dulbecco's modified Eagle medium DMEM (w/o phenol red) supplemented with glutamine, penicillin–streptomycin, 10% (v/v) FBS and 2 μg ml^{-1} puromycin. Cells were grown in 10% FBS until judged to 50–60% confluent and serum starved by aspiration of medium, washing once with serum-free medium and then replacing with fresh serum-free medium. 100 nM 4-HT was added to activate the appropriate kinase:ER* fusion and the equivalent volume of ethanol was employed as a vehicle control. Cells were then harvested at the indicated times.

2.3. Western blotting

At the required times, cells were washed in phosphate buffered saline (PBS) and harvested on ice in lysis buffer (20 mM Tris [pH7.5], 137 mM NaCl, 1mM EGTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1.5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM PMSF, 20 μM leupeptin, 10 μg ml^{-1} aprotinin and 50 mM NaF). Cell extracts were snap frozen, thawed and cleared by centrifugation. Supernatant protein concentrations were measured by Bradford protein assay (Bio-Rad) and equal quantities of cell extracts were resolved by SDS-PAGE and transferred to Immobilon P membranes (Millipore). Membranes were then blocked for at least 1 h in 0.1% (v/v) Tween-20/PBS containing 5% (w/v) powdered milk before being probed with the desired antibodies (see Materials). Immune-reactive proteins were visualised with the enhanced chemi-luminescence (ECL) system (Amersham).

2.4. Immune complex kinase assays

Following stimulation, cells were washed in cold PBS, harvested as described above and assayed for ERK1, JNK1 and p38α as described previously [7,28,29]. Incorporation of [^{32}P] into respective substrates was quantified by PhosphorImager (Fuji).