

## The effect of C-terminal fragment of JNK2 on the stability of p53 and cell proliferation

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### ABSTRACT

The basal activity of JNK is low in normal growing cells and inactivated JNK targets p53 for ubiquitination. To elucidate if the C-terminal part of JNK is responsible for its binding to p53, the low background tet-off inducible NIH3T3 cell line was selected by luciferase reporter gene and a double stable C-JNK Aa (203-424) cell line was established. After withdrawing tetracycline, the C-JNK fragment expression was induced and cell growth was dramatically inhibited 24 h later. However, the expression of p53 was found to be increased after the induction of C-JNK fragment, evaluated by transfecting p21<sup>waf</sup>-luciferase reporter genes. Our further studies showed that C-JNK fragment could form complex with p53 both *in vivo* and *in vitro*. Induction of C-JNK fragment *in vivo* can increase p53 stability by inhibiting p53 ubiquitination.

**Keywords:** tet-off expression system, c-Jun N-terminal kinase, p53, cell proliferation.

### INTRODUCTION

The superfamily of mitogen-activated protein kinase (MAPK) consists of evolutionarily conserved proteins which play an important role in the regulation of intracellular metabolism and gene expression, therefore they are correlated with a variety of cellular functions including cell growth, differentiation, apoptosis as well as cellular response to environmental stresses and pro-inflammatory cytokines. MAPK signal transduction pathways have been proved to be ubiquitous in eukaryotes [1-5]. As major components, there are three Ser/Thr kinases within the MAPK family: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase (JNKs)/stress-activated protein kinases (SAPKs) and p38 MAPKs [6, 7]. ERKs are activated preferentially by mitogens and EGF [7, 8], while JNK and p38 pathways are stimulated mainly by proinflammatory cytokines and diverse array of cellular stresses [9-12].

JNK plays a pivotal role in cellular stresses such as UV light, heat, anisomycin, hydrogen peroxide or hyperosmolarity. In normal growing cells, the efficiency of JNK is limited due to the influence of glutathione S-transferase pi (GSTp), a natural inhibitor of JNK [13]. Under this condition, JNK mediates the ubiquitination and degradation of its associated proteins such as c-Jun, ATF2 and p53 [14-17]. When responding to environmental stress, JNK is activated by dual phosphorylation at the Thr-Pro-Tyr motif by up-stream kinases MKK4/7, leading to the phosphorylation of its substrates such as c-Jun [18, 19], ATF2 [20], c-Myc [21], Bcl-2 [22] and p53 [23], and then breaks cellular balance.

So far, ERKs have been profoundly studied [24], but the respective functions of JNK and its domains remain unclear. Several reports demonstrated that the effect of JNK on p53 in both normal cells and those exposed to external stresses. The association of JNK with p53 could efficiently inhibit p53 ubiquitination and this process is Mdm2 independent [15]. In order to elucidate the regulation of p53 by effective domains of JNK, a NIH 3T3/Tet-off inducible expression system was established, and the C-JNK (aa 203-424) positive clones were selected by G418 and hygromycin. By removing tetracycline, we studied its

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effect on stability and transcription activity of p53 as well as cell growth.

## MATERIALS AND METHODS

### Cells lines

The mouse fibroblast cell line NIH 3T3 that stably expresses the pSV40-Hyg plasmid (Clontech) was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>.

### The selection of high expression and low basal level NIH3T3 cells

NIH 3T3 cells plated in a 6-well plate at 3×10<sup>5</sup> cells/well was changed to DMEM containing 1 µg/ml tetracycline (tet) (Calbiochem) 24 h prior to the experiment. Luciferase-pUHD-10-3 reporter vector (0.5 µg) as a gift from Dr. Bujard H (University of Heidelberg, Germany) was incubated with LipofectAMINE (Invitrogen) for 15 min and then was added into each well. After 24 h incubation, tet was removed and 8 h later cells were harvested for quantitation of activity of luciferase.

### Establishment of double stable C-JNK cell lines and fragment expression

The pTet-C-JNK was constructed by subcloning the cDNA of the C-terminal 222 amino acids of wild type JNK2 into the pUHD-10-3 vector. The C-JNK stable clones were selected in 600 µg/ml geneticin (Calbiochem) in the presence of hygromycin (100 µg/ml) after transfection. To suppress the expression of C-JNK, tet (final concentration 1 µg/ml) was added to the medium every 3 d. While in order to turn on the expression of C-JNK, cells were washed 3 times with PBS to remove tet and allowed to incubate in DMEM supplemented with 10% fetal bovine serum.

### Immunoprecipitation (IP) and immunoblots

Polyclonal antibodies for JNK and p53 were generated by using bacterially expressed JNK and p53 as antigens. Anti-p21 mAb was from Labvision company. For immunoprecipitation, whole cell extract (WCE) was incubated with the above antibodies for 16 h at 4 °C respectively, and then treated by 20 ml protein G-beads (Invitrogen) for 30 min at room temperature. Following 4 times intensive wash with PBS/0.5M LiCl supplemented with Tween 100 (0.5%), the extract was subjected to Western blot analysis.

Immunoblot analysis was performed as follows: 50-100 µg of WCE separated by SDS-PAGE and then electrotransferred to a nitrocellulose membrane. After ponceau staining to confirm equal loading, the membrane was blocked by 5% non-fat milk, washed with TBST for 4 times, and incubated with anti-HA, anti-p53, or anti-p21<sup>waf</sup> monoclonal antibodies as indicated and then the HRP-conjugated goat anti mouse IgG antibody (Calbiochem) for 1 h each. Results were visualized using ECL (Amersham Pharmacia Biosciences).

### Cell proliferation detection

NIH3T3 double stable cells cultured in 6-well plates were induced for the expression of JNK by removing tet. After 24 h, tet was added back to inhibit C-JNK expression. To determine cell

proliferation, cells in each well were incubated with 1 µCi <sup>3</sup>H-TdR for 24 h and then harvested. The radioactivity of <sup>3</sup>H-TdR incorporation into DNA was measured using liquid scintillography (Perkin Elmer).

### In vitro ubiquitination assay

For *in vitro* ubiquitination assay, 50 µg of whole cell lysates prepared from C-JNK expressing cells that were incubated with bacterially expressed human tagged his-p53 (5 µg on ice) and then with NTA beads for 1 h. After intensive washes, the substrate bound beads were equilibrated with ubiquitin buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 2 mM NaF and 3 µM okadaic) and incubated in the same buffer supplemented with 2 mM ATP, 10 mM creatine phosphate, 0.02 unit of creatine phosphokinase, 2 µg of hemagglutinin (HA)-tagged ubiquitin, 1.5 mM [ $\gamma$ -<sup>32</sup>P]ATP (Sigma) and 33% reticulocyte lysate(v/v) (Promega) in a total volume of 30 µl at 37 °C for 5 min.

## RESULTS

### Establishment of double stable C-JNK cell lines and fragment expression

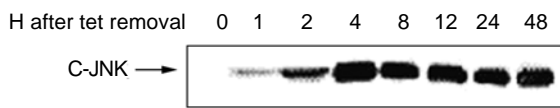
The HA-C-JNK cDNA inserted pUHD-10-3 plasmid was transfected into the cells with high expression and low basal level and tet was added immediately to a final concentration of 1 µg/ml. Resistant clones were selected by using 600 µg/ml geneticin (G418) and 100 µg/ml hygromycin. There is an exogenous HA tag at the N-terminus of C-JNK for avoiding the endogenous JNK, so when tet (1 µg/ml) was present, C-JNK fragment expression was suppressed. On the other hand, if tet was withdrawn by three times washing with PBS and adding normal medium, the expression of C-JNK increased with time, as detected by Western blot (Fig. 1). As a control, GST protein level during such period kept stable (data not shown).

### The effect of C-JNK fragment induction on cell proliferation

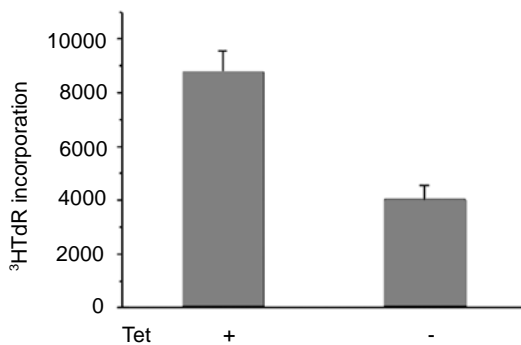
The radioactivity of <sup>3</sup>H-TdR was measured to determine cell proliferation when tet was added back 24 h after being removed to stop further expression. We showed that cell proliferation decreased obviously compared to the control cells with the induction of C-JNK fragment (Fig. 2). This result is consistent with the finding that forced expression of C-JNK fragment increased p21<sup>waf</sup>-luciferase activity (Fig. 4 A) and p53 stability (Fig. 3A).

### Decrease of p53 ubiquitination and its upregulation after forced expression of C-JNK fragment

As shown in Fig. 3A, after induction of C-JNK fragment, the endogenous p53 protein was obviously up-regulated. In order to determine whether such increase of p53 protein level was resulted from the inhibition of ubiquitination of



**Fig. 1** Expression of C-JNK in NIH 3T3 cells after removal of tetracycline. HA-tagged C-JNK was used to established 3T3 cells that are tet-regulated. Mixed populations of cells were selected based on drug resistance and analyzed for C-JNK expression at the indicated time points after tet removal. Western blotting analysis revealed the expression of a 25 kD protein detected by anti-HA.

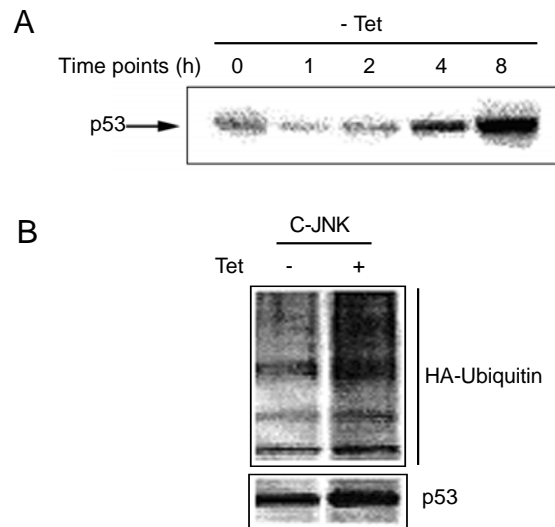


**Fig. 2** <sup>3</sup>H-TdR incorporation into DNA before and after C-JNK induction. C-JNK expressing NIH 3T3 cells ( $10^5$  in 6-well plates) were cultured in the presence or absence of tet. Cells were prepared for analysis 24 h after <sup>3</sup>H-TdR being added. Each analysis was performed in triplicate. The data represents three independent experiments.

p53, the degree of smear above p53-reacting band representing polyubiquitin chains of HA-ubiquitin was detected to describe changes in the level of p53 ubiquitination according to C-JNK expression. Results showed the ubiquitin bound to p53 was evidently reduced with the expression of C-JNK (Fig. 3B), indicating that C-JNK may be involved in the ubiquitination of p53.

### The effect of forced expression of C-JNK fragment on p53

p21<sup>waf</sup>, which can be activated by p53 and lead to cell apoptosis or growth arrest, was used to measure p53 activity. In order to study the effect of forced expression of C-JNK fragment on p53, p21<sup>waf</sup>-luciferase reporter vector was transfected into the selected cells. Then the expression of C-JNK fragment was induced by removing tet, the luciferase activity were measured before and after induction. Our result showed clearly that p53 activity increased after the induction of C-JNK fragment, which is

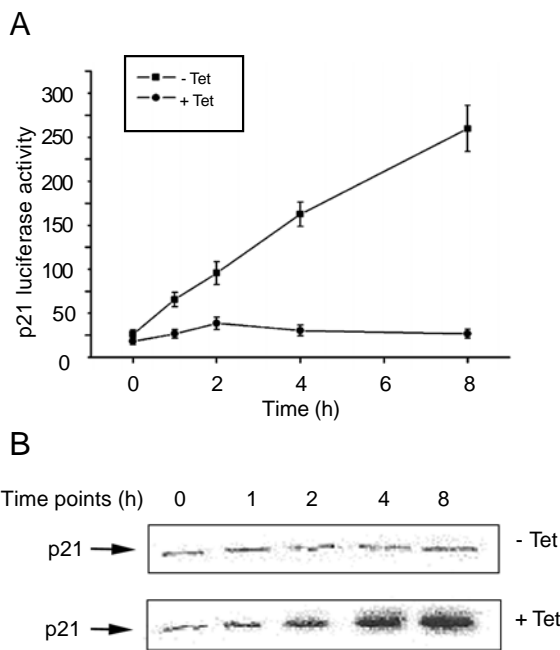


**Fig. 3** (A) The effect of C-JNK on p53 protein level. Cells expressing C-JNK were maintained in tet-free medium and harvested at indicated time points. Western blot analysis was carried out using monoclonal pAb421 antibodies against p53. (B) C-JNK effect on *in vitro* ubiquitination of p53. Ubiquitination of bacterially expressed purified p53 incubated with cell lysates that either contained or lacked C-JNK fragment was carried out as described under “Material and Methods”. A high molecular weight smear was detected by antibodies to HA recognized HA-ubiquitin, which formed a poly-ubiquitin chain covalently bound to p53.

supported not only by elevated p21<sup>waf</sup>-luciferase activity, but also the p21 protein level (Fig. 4).

### Association of C-JNK and p53 *in vitro* and *in vivo*

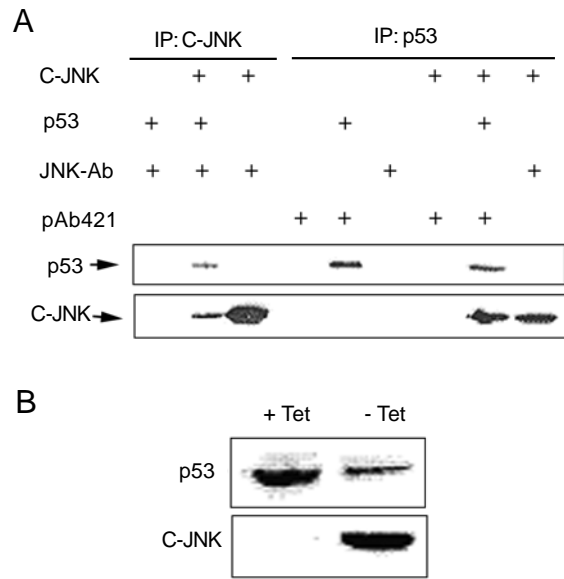
The inducible C-JNK system and the bacterially expressed p53 and C-JNK were used to further detect if C-JNK could efficiently interact with p53. For *in vitro* studies, bacterially expressed his-tagged p53 protein was incubated with beads-bound HA-C-JNK. As shown in fig. 5A, co-precipitating p53 was detected by immuno-blot analysis with anti-p53 antibody (Fig. 5A lanes 1-3). Reciprocal experiments showed C-JNK was also detected in the nickel-beads-bound his-p53 pull-down complexes (Fig. 5A lanes 4-9). *In vivo* experiment of measuring the association between C-JNK and p53 was demonstrated in cells expressing inducing C-JNK, using anti-p53 antibody to immunoprecipitate p53. Fig. 5B shows that C-JNK was detected in tet-induced cells, whereas absent in non-induced cells. These results suggested that JNK had some docking sites for p53 association.



**Fig. 4 (A)** Transcriptional activates of p53 in C-JNK-expressing cells. Cells ( $10^5$  in 6-well plates) were cotransfected with p21<sup>waf</sup>-luciferase and  $\alpha$ -gal constructs (0.5  $\mu$ g of each) and maintained in presence or absence of tet. After 24h, cells were harvested at the indicated time points. Luciferase assays were carried out using proteins normalized as per  $\alpha$ -gal activates to reflect equal transfection efficiency. Data shown represents three experiments performed in duplicates. **(B)** The p21 protein level at different time points before and after removing tet. Cells were harvested at different time points before and after removing tet. The level of p21 levels were detected by western blot using p21 antibody.

**DISSCUSSION**

The most common ones among mechanisms underlying the regulation of stress kinases are their dimerization and abrogation of intramolecular inhibition, both of which are dependent upon their phosphorylation by each respective upstream kinase [24]. However, for JNK, the characters and functions of its domains were still kept rarely informed. This study aims to elucidate which part of JNK is responsible for the binding of p53, by using the low background tet-off inducible NIH3T3 cell line selected by luciferase reporter gene and a double stable C-JNK Aa (203-424) cell line. The finding that the induction of C-JNK fragment could lead to JNK phosphorylation (data not show) hinted that C-JNK opened the phosphorylation sites of JNK, as similar as other MAPKs. In normal growing cells, one of the mechanisms to keep low base level of p53 in normal cells lies in the binding of inactivated JNK to p53, which in turn leads to the degradation of p53 [15].



**Fig. 5 (A)** *In vitro* association of bacterially expressed C-JNK with p53. Bacterially expressed his-p53 and HA-C-JNK were purified on nickel or HA-antibody-coupled beads, respectively. Soluble C-JNK was incubated with the bead-bound p53 for 15 min on ice before beads were subjected to three washes. Antibodies to either p53 or to HA were used to immunoprecipitate respective complexes. Immunoprecipitated material was washed and analyzed by western blots using HA or p53 antibodies as indicated. **(B)** *In vivo* association of immunoprecipitated p53 with C-JNK. C-JNK expressing cells were grown in the presence or absence of tet. Immunoprecipitation was carried out using monoclonal pAb421 antibodies against p53. Bound C-JNK was detected using HA antibodies which identified HA-tagged C-JNK. Data shown here represents three independent analyses.

Previous studies revealed that wide type JNK contributes to the stability as well as the activity of p53 [14,15]. In the present study, our findings demonstrated that the forced expression of C-JNK fragment obviously inhibited cell proliferation. So next we focus on whether C-JNK could regulate p53. We found that C-JNK fragment could form complex with p53 both *in vivo* and *in vitro* and the induction of C-JNK fragment *in vivo* can increase p53 stability by altering p53 ubiquitination. It is obvious that the forced expression of C-JNK fragment could stabilize p53, and furthermore, the induction of C-JNK fragment could affect cell cycle.

What we found in the function of C-JNK fragment in inhibiting p53 stability is quite similar with that of N-JNK fragment [25], which may hint that both terminals of JNK hold sites for p53 association. The difference between N-JNK and C-JNK fragment is that induction of N-JNK fragment increases the sensitivity of cells to H<sub>2</sub>O<sub>2</sub> while

induction of C-JNK regulates cell proliferation [25]. Although the docking sites of c-Jun and p53 for JNK are clear [16, 26], which domain of JNK is crucial for the association of p53 and c-Jun still remains unknown. Our results suggest that both N-JNK and C-JNK are responsible for full length JNK to bind with p53.

MDM2 is another cellular protein which mediates p53 ubiquitination and degradation. The relationship between C-JNK and MDM2 should be carried out in our further studies.

## ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (No.30270556) and The National Basic Research Program (No.2002CB513004). We would also like to thank Dr. Ze'ev Ronai (Department of Oncological Sciences, Mount Sinai School of Medicine, NY, USA) for the constructions of some plasmids and cell lines.

Received: Jul 11, 2003

Revised: Jun 7, 2004

Accepted: Jun 15, 2004

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