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Novel role of c-jun N-terminal kinase in regulating the initiation of cap-dependent translation

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Abstract. Initiation of protein translation by the 5' mRNA cap is a tightly regulated step in cell growth and proliferation. Aberrant activation of cap-dependent translation is a hallmark of many cancers including non-small cell lung cancer. The canonical signaling mechanisms leading to translation initiation include activation of the Akt/mTOR pathway in response to the presence of nutrients and growth factors. We have previously observed that inhibition of c-jun N-terminal kinase (JNK) leads to inactivation of cap-dependent translation in mesothelioma cells. Since JNK is involved in the genesis of non-small cell lung cancer (NSCLC), we hypothesized that JNK could also be involved in activating cap-dependent translation in NSCLC cells and could represent an alternative pathway regulating translation. In a series of NSCLC cell lines, inhibition of JNK using SP600125 resulted in inhibition of 4E-BP1 phosphorylation and a decrease in formation of the cap-dependent translation complex, eIF4F. Furthermore, we show that JNK-mediated inhibition of translation is independent of mTOR. Our data provide evidence that JNK is involved in the regulation of translation and has potential as a therapeutic target in NSCLC.

Introduction

Initiation of 5' cap-dependent protein translation is a highly regulated cellular process that is finely tuned to the metabolic needs of a cell. We and others have shown that aberrant activation of the cap-dependent translational machinery is important to the survival of cancer cells, including NSCLC (1-3). Furthermore, in many cancer cells, the negative regulator of cap-dependent translation, eukaryotic initiation factor 4E binding protein 1 (4E-BP1) is found to be hyperphosphorylated and thereby unable to sequester eukaryotic initiation factor 4E (eIF4E) and inhibit the translation of key growth and anti-apoptotic proteins (4,5). The canonical pathway leading to phosphorylation of 4E-BP1 and initiation of cap-dependent translation occurs via activation of receptor tyrosine kinases by growth factor signaling through Akt/mammalian target of rapamycin (mTOR), which phosphorylates 4E-BP1. This induces a conformational change in 4E-BP1 allowing the release of eIF4E, freeing it to associate with eIF4G leading to recruitment of ribosomes and activation of translation (6). Since aberrant activity of translation correlates with poorer outcomes in cancer, there has been increasing interest in understanding this pathway and signaling events regulating translation in cancer cells (1,7).

4E-BP1 contains 6 phosphorylation sites which undergo hierarchical phosphorylation. Each phosphorylation event reduces the affinity of 4E-BP1 for eIF4E. mTOR has been shown to phosphorylate 4E-BP1 at Thr-37 and Thr-46 which has been seen as a priming event for full phosphorylation by as yet unidentified kinases, which is required for release of 4E-BP1 from eIF4E (8). 4E-BP1 can serve as a substrate for several kinases *in vitro* including c-Jun N-terminal kinase (JNK) and extracellular-regulated kinase (ERK) 2, however, in its physiologic state bound to eIF4E many of the phosphorylation sites are unavailable. Only mTOR has been shown to phosphorylate it in this state (9). Thus, it has been concluded that mTOR phosphorylation causes a conformational shift in 4E-BP1 allowing other kinases to phosphorylate 4E-BP1 on the remaining sites and lead to full activity of the cap-complex (8). We recently published results showing that c-jun N-terminal kinase (JNK) inhibition by SP600125 led to decreased phosphorylation of 4E-BP1 and decreased initiation of cap-dependent translation in mesothelioma cells (10). Interestingly, several publications have demonstrated that JNK plays an important role in lung carcinogenesis (11-13). We were interested to further study the role of JNK activation in regulating cap-dependent translation in NSCLC cells and gain further insight into the signaling pathways involved. Here we show that JNK is activated in

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several NSCLC cell lines and SP600125, a JNK inhibitor, leads to decreased phosphorylation of 4E-BP1 in a dose-dependent manner. Furthermore, we show that this decrease in phosphorylation results in decreased formation of the eIF4F initiation complex and this effect is independent of mTOR. These data suggest that regulation of the initiation of cap-dependent protein translation is a novel function of JNK signaling.

Materials and methods

Cell lines and reagents. Cell lines were purchased from the ATCC or provided by Frederick Kaye (NIH). H2009, H522, H520, H460, H1299, H2030, H661 NSCLC cells were grown in RPMI-1640 supplemented with 10% fetal calf serum and 1% penicillin/streptomycin/fungicin antibiotic (2). Normal human bronchial epithelial cells (NHBE) were grown in BEGM medium (BEBM supplemented with SingleQuots, Cambrex Biosciences) as described previously (2). For treatment experiments, the JNK inhibitor, SP600125, and rapamycin were purchased from Calbiochem. Both drugs were dissolved in 100% DMSO and stored at -20˚C. Cells were treated with various concentrations of drugs as well as controls treated with an equal volume of the drug's vehicle, DMSO.

Kinase assays. JNK activity was determined by *in vitro* kinase assay kit (Cell Signaling) using the manufacturer's instructions as described (10). Cells were grown to 70% confluence and lysed. Lysate (300 μg) was added to 30 μl of c-Jun sepharose beads and rotated overnight at 4˚C to pull-down JNK. Beads were washed with lysis buffer thrice and incubated in kinase buffer supplemented with 200 μM ATP for 30 min at 30˚C. Beads were eluted using 3X loading buffer and run on SDS-PAGE gels and subjected to Western blotting for phospho-c-jun.

Immunoblot analyses. Gels were transferred onto PVDF membranes, blocked in 5% milk-Tris-buffered saline with Tween-20 (TBST) for 1-2 h at room temperature. Primary antibodies were diluted in TBST and incubated overnight at 4˚C. For phospho-specific antibodies, the blocking and primary antibody dilutions were in 5% BSA-TBST. Anti-JNK, anti-phospho-c-Jun (Ser63) (1:500 dilution), anti-eIF4E, anti-phospho-eIF4E (Ser209), anti-4E-BP1, anti-phospho-4E-BP1 (Ser65), anti-p70S6 kinase, anti-phospho-p70S6 kinase (Thr389), anti-Akt, anti-phospho-Akt (Ser473), anti-poly-ADP-ribose polymerase (PARP), anti-Bcl-2, and anti-c-Myc antibodies were obtained from Cell Signaling. Anti-Bcl-xL was obtained from Santa Cruz Biotechnology. Rabbit anti-eIF4G (diluted at 1:2,000) was provided in kind by Nahum Sonenberg (10,14). All antibodies were diluted 1:1,000 except where indicated. Western blots were analyzed for optical density using NIH Image J software.

Cell proliferation assay. Cells were seeded in triplicate onto 96-well plates with 2,000 cells/well along with controls. Cells were treated with SP600125 24 h after plating. All samples and controls were treated with equal volume of the drug vehicle, dimethyl sulfoxide (DMSO), and grown for 72 h. Cell viability was measured using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies) following the manufacturer's protocol. The amount of CCK-8 reagent reduced to formazan by cellular dehydrogenase indicating cell viability was assayed by reading the absorbance at 405 nm on a 96-well plate reader. Statistical analysis was performed by the t-test. A $p<0.05$ was significant.

Cap-binding assay. The cap-binding assay was carried out as previously described (10). Cells grown to 70% confluence were lysed using 1X cell lysis buffer (Cell Signaling). Protein lysate (300 μg) was added to 50 μl of 50% slurry of 7^{m}GTP-sepharose beads to isolate eIF4E and its binding partners 4E-BP1 and eIF4G. Lysates were rotated at 4˚C for 2 h followed by 3 washes in lysis buffer. Proteins bound to the beads were eluted by adding 50 μl of 5X Laemmli's buffer and boiling at 95˚C for 5 min. The eluate was analyzed by Western blotting for eIF4E, 4E-BP1, eIF4G, and JNK.

Co-immunoprecipitation. Antibodies for immunoprecipitation, anti-4E-BP1 and anti-JNK antibodies, were obtained from Cell Signaling and Millipore, respectively. Protein (300 μg) was diluted in 1X lysis buffer to a concentration of 1 μg/μl and antibody was added to each tube to make a final dilution of 1:50. Samples were rotated at 4˚C overnight followed by 5-h incubation with protein-A sepharose beads. Samples were centrifuged at 14,000 rpm for 30 sec and supernatant was removed. Beads were washed thrice with 500 μl of 1X lysis buffer and eluted with 3X Laemmli's buffer and subjected to 10% SDS-PAGE.

Results

A series of NSCLC cell lines were cultured under standard conditions and lysates from these cells were subjected to *in vitro* kinase assays to detect activation of JNK. Normal human bronchial epithelial cells (NHBE) were used as a control. As shown in Fig. 1A, JNK activity was at least 1.5-fold greater in 4 of 7 NSCLC cells compared to NHBE cells. 4E-BP1 can be resolved into 3 isoforms based on electrophoretic mobility: α, β, and γ; indicating hypo-, intermediate, and hyper-phosphorylated isoforms, respectively (15). Successive phosphorylation of 4E-BP1 causes a slowing of its electrophoretic mobility such that it is detectable as a separate band on a Western blot. Compared to NHBE cells, 4E-BP1 was predominantly in the hyper-phosphorylated form in the majority of NSCLC cell lines examined, in accord with reported results (2). Since we had previously shown that JNK inhibition leads to decreased phosphorylation of 4E-BP1 in mesothelioma (10), we further studied the effect of JNK inhibition using a specific inhibitor, SP600125, on 4E-BP1 phosphorylation in NSCLC. SP600125 decreased JNK activity as assessed by *in vitro* kinase assay in all 4 cell lines (Fig. 1B). In H2009 cells, JNK activity returned to basal levels when treated with 25 μM SP600125. Because the kinase assay involves pull-down of c-jun and its binding partners, it is possible that this result reflects pull-down and phosphorylation of c-jun by an alternate kinase (16), however, this is speculative. SP600125 appeared to specifically target JNK at these doses as extracellular-regulated kinase (ERK) phosphorylation was not decreased in any of the cell lines. In the 4 NSCLC cell lines that showed increased JNK activity, JNK inhibition decreased phosphorylation (less γ isoform) of 4E-BP1 in a dose-dependent manner (Fig. 1C), a finding that was confirmed using a phospho-specific antibody to $4E\text{-}BP1^{Ser65}$.

Since hypophosphorylated 4E-BP1 binds eIF4E and prevents eIF4F formation, we examined the effects of JNK inhibition

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