COOH-Terminal Src Kinase–Mediated c-Jun Phosphorylation Promotes c-Jun Degradation and Inhibits Cell Transformation

Feng Zhu, Bu Young Choi, Wei-Ya Ma, Zhongliang Zhao, Yiguo Zhang, Yong Yeon Cho, Hong Seok Choi, Akira Imamoto, Ann M. Bode, and Zigang Dong

Abstract

The oncprotein c-Jun is a component of the activator protein-1 transcription factor complex, which is involved in cellular proliferation, transformation, and death (1). One of the mechanisms controlling the activation of AP-1 is the regulation of the activity of the various AP-1 protein components, particularly c-Jun. c-Jun is the most prevalent constituent of the AP-1 transcription factor complex, and its mRNA and protein expression are induced in response to various extracellular signals (2). However, following exposure to phorbol esters, growth factors, and transforming oncogenes, posttranslational modifications, especially phosphorylation, of c-Jun may be more important (3). Thus, the identification of kinases responsible for c-Jun phosphorylation is essential. Currently, several kinases have been found that can phosphorylate c-Jun. These kinases include c-Jun NH2-terminal kinases (JNKs; ref. 4), p34cdc2, extracellular signal-regulated protein kinases (ERKs), protein kinase C (PKC; ref. 5), CKII (6), GSK3 (7), DNA-PK (8), and c-Abl (9). Among these kinases, phosphorylation by JNKs is currently believed to be the most important positive regulator of c-Jun activity and cell transformation caused by c-Jun. These results indicated that this function of CSK controls cell proliferation under normal growth conditions and may have implications for CSK loss of function in carcinogenesis. (Cancer Res 2006; 66(11): 5729-36)

Introduction

The activator protein-1 (AP-1) transcription factor is involved in cellular proliferation, transformation, and death (1). One of the mechanisms controlling the activation of AP-1 is the regulation of the activity of the various AP-1 protein components, particularly c-Jun. c-Jun is the most prevalent constituent of the AP-1 transcription factor complex, and its mRNA and protein expression are induced in response to various extracellular signals (2). However, following exposure to phorbol esters, growth factors, and transforming oncogenes, posttranslational modifications, especially phosphorylation, of c-Jun may be more important (3). Thus, the identification of kinases responsible for c-Jun phosphorylation is essential. Currently, several kinases have been found that can phosphorylate c-Jun. These kinases include c-Jun NH2-terminal kinases (JNKs; ref. 4), p34cdc2, extracellular signal-regulated protein kinases (ERKs), protein kinase C (PKC; ref. 5), CKII (6), GSK3 (7), DNA-PK (8), and c-Abl (9). Among these kinases, phosphorylation by JNKs is currently believed to be the most important positive regulator of c-Jun activity. However, identifying kinases that can phosphorylate c-Jun resulting in its down-regulation could be very helpful in understanding and suppressing the mechanism of neoplastic transformation that is caused by c-Jun.

The most well-characterized phosphorylation sites of c-Jun are Ser63 and Ser73 near the NH2 terminus (10). The phosphorylation of c-Jun by JNKs prevents the ubiquitin-dependent degradation of c-Jun, and this phosphorylation-dependent stabilization contributes to the efficient activation of c-Jun following exposure to growth factors, stress, or other stimulators of c-Jun activity (11). Currently, JNK2 is the only known kinase that functions as a negative regulator of the ubiquitin-dependent degradation of c-Jun under normal growth conditions (12). On the other hand, the positive regulation of the ubiquitin-dependent degradation of c-Jun may be critical in maintaining a low steady-state level of the c-Jun protein, which could result in the suppression of AP-1 activity and inhibition of c-Jun-caused cell transformation.

In this study, we identified COOH-terminal Src kinase (CSK) as a novel c-Jun kinase. Phosphorylation of c-Jun by CSK, in contrast to JNK1 and ERKs, was found to be a positive regulator of the ubiquitin-dependent degradation of c-Jun, thereby inhibiting c-Jun function under physiologic growth conditions. Our results indicated the existence of a new signaling route for maintaining a low steady-state level of c-Jun and thus, for controlling cell proliferation under normal growth conditions.

Materials and Methods

Plasmids and cell lines. Seventy-two kinases were isolated from the A549 cell line by RT-PCR and cloned into the pJASPl vector and confirmed by sequencing. The MT35 (His6-tagged c-Jun) plasmids were kindly provided by Dr. Dirk Bohmann (Department of Biomedical Genetics, University of Rochester, Rochester, NY; ref. 11). Csk-wt was kindly provided by Dr. Gordon Langsley (Laboratoire de Signisation Immunoparasitaire, Department d’Immunologie, Institute Pasteur, Paris, France; ref. 13). Site-directed mutagenesis was done using the Quickchange kit (Stratagene, Cedar Creek, TX). Mutated c-Jun plasmids were subcloned into pcDNA4-HisMA or into pGEX-5x-1 vectors. The expression of recombinant proteins was induced in Escherichia coli BL21 at 30°C for 0.5 to 4 hours by the addition of 0.2 mmol/L isopropyl-1-thiogalactopyranoside. Glutathione S-transferase (GST)–tagged proteins were affinity-purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ). His-tagged proteins were purified using Ni-NTA agarose (Qiagen, Valencia, CA) after transient transfection with Superfect (Qiagen).

Antibodies and coimmunoprecipitation. The c-Jun antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and the phospho-tyrosine and CSK antibodies were purchased from Upstate Biotechnology, Inc. (Charlottrsville, VA). Anti-phospho-Src-528 was purchased from Cell Signaling, Inc. (Beverly, MA). For coimmunoprecipitation, cell lysates were clarified by centrifugation at 4°C after adding 1.0 µg of the control IgG and 20 µL of agarose-conjugated protein A-G beads. The supernatant fractions were transferred to new tubes and 2 µg of selected primary antibodies were added and incubated overnight.

Note: The University of Minnesota is an equal opportunity educator and employer.

Requests for reprints: Zigang Dong, Hormel Institute, University of Minnesota, 801 16th Avenue Northeast, Austin, MN 55912. Phone: 507-437-9600; Fax: 507-437-9600; E-mail: zgdong@hi.umn.edu.

© 2006 American Association for Cancer Research.
induced cell colonies were scored by the methods described (14). Cultures were maintained in a 37°C 0.5% BME agar containing 10% fetal bovine serum medium. The cultures were prepared and the expression of c-Jun was analyzed by immunoblotting. A

Anchorage-independent transformation assay. A

**Results**

CSK phosphorylates c-Jun in vitro. To identify novel kinases that phosphorylate c-Jun, 72 kinases were cloned and each was individually inserted into the pBIND vector and c-jun fusion proteins or peptides (1.5 μg) were mixed, respectively, together with CSK in 0.5 mmol/L ATP and 1× kinase buffer, and then incubated at 30°C for 15 minutes. Samples were separated by SDS-PAGE followed by autoradiography or Western blot analysis. The peptides were synthesized by Invitrogen (Carlsbad, CA).

**In vitro kinase assay.** Active CSK and 10× kinase buffer were purchased from Upstate Biotechnology. c-jun fusion proteins or peptides (1.5 μg) were incubated with CSK in 0.5 mmol/L ATP and 1× kinase buffer, and then incubated at 30°C for 15 minutes. Samples were separated by SDS-PAGE followed by Western blotting.

**In vitro ubiquitination assay.** The Fraction II HeLa Conjugation Kit was purchased from Boston Biochem, Cambridge, MA. In brief, 17 μL of Fraction II HeLa HeLa (200 μg), 1.25 μL of MG-132 (5 μmol/L), and 2 μL of ubiquitin aldehyde were added in each reaction tube, vortexed, and the inhibitor and Fraction II HeLa were allowed to preincubate for 15 minutes at room temperature. Next, 5 μL of the ubiquitin solutions were added into tubes for in vitro ubiquitination, but not into control tubes. The GST-c-Jun or GST-c-Jun mutant (c-Jun-FY26170FF) was added into control or experimental tubes, respectively, and the conjugation reaction was initiated with the addition of 5 μL of the energy regeneration solution. Samples were vortexed and incubated at 37°C for 1 to 2 hours. The samples were analyzed by Western blot using an ubiquitin antibody.

**Pulse-chase analysis and cycloheximide chase analysis.** CSK+/cells and CSK−/− cells were plated the day before the experiment. On the day of the experiment, the medium was aspirated from each plate of cells. Cells were washed twice in 5 mL warm PBS. Warm DMEM (1 mL, met/cys-free) /C2 was added into each reaction tube, vortexed, and the inhibitor and Fraction II HeLa were allowed to preincubate for 15 minutes at room temperature. Next, 5 μL of the ubiquitin solutions were added into tubes for in vitro ubiquitination, but not into control tubes. The GST-c-Jun or GST-c-Jun mutant (c-Jun-FY26170FF) was added into control or experimental tubes, respectively, and the conjugation reaction was initiated with the addition of 5 μL of the energy regeneration solution. Samples were vortexed and incubated at 37°C for 1 to 2 hours. The samples were analyzed by Western blot using an ubiquitin antibody.

Pulse-chase analysis and cycloheximide chase analysis. CSK+/− cells and CSK−/− cells were plated the day before the experiment. On the day of the experiment, the medium was aspirated from each plate of cells. Cells were washed twice in 5 mL warm PBS. Warm DMEM (1 mL, met/cys-free) /C2 was added into each reaction tube, vortexed, and the inhibitor and Fraction II HeLa were allowed to preincubate for 15 minutes at room temperature. Next, 5 μL of the ubiquitin solutions were added into tubes for in vitro ubiquitination, but not into control tubes. The GST-c-Jun or GST-c-Jun mutant (c-Jun-FY26170FF) was added into control or experimental tubes, respectively, and the conjugation reaction was initiated with the addition of 5 μL of the energy regeneration solution. Samples were vortexed and incubated at 37°C for 1 to 2 hours. The samples were analyzed by Western blot using an ubiquitin antibody.

Csk and CSK bind with and phosphorylates c-Jun in vitro. A, the interaction between the two test proteins, as GAL4-CSK (pBIND-csk) and VP16-c-Jun (pACT-c-jun) fusion constructs (lane 4), resulted in an increase in luciferase expression compared with the negative controls (lanes 1, 2, and 3). JNK1 (pBIND-jnk1; lane 5) was used as a positive control. Columns, mean of three independent experiments; bars, ± SD. Significant differences were evaluated using Student’s t test (*, P < 0.01). B, CSK phosphorylates c-Jun in the in vitro kinase assay in the presence of 32P as visualized by autoradiography. C, CSK phosphorylates c-Jun at tyrosine sites as determined by an in vitro kinase assay with detection by Western blotting.
CSK phosphorylated c-Jun at tyrosine sites (Fig. 1C). These same samples were used in additional Western blots to determine whether CSK phosphorylates c-Jun at Ser<sup>63</sup>, Ser<sup>73</sup>, or other serine/threonine sites. In this case, no signal was detected (data not shown), confirming the specificity of CSK for tyrosine phosphorylation of c-Jun in vitro.

**CSK phosphorylates c-Jun in vivo.** Subsequently, using NIH3T3 cells, we tested whether c-Jun and CSK interacted with each other in vivo. In the mammalian two-hybridization system, c-Jun and CSK were both transported into the nucleus after translation because of the nuclear localization sequence present in the experimental GAL4 or VP16 tags. Therefore, testing whether c-Jun and CSK have a similar localization in cells (in vivo) is important to eliminate the possibility of a false-positive result. Localization experiments indicated that c-Jun and CSK were located both in the cytosol and the nucleus (Fig. 2A). The similar localization of c-Jun and CSK meant that the possibility exists for them to bind with each other in vivo. This possibility was confirmed with immunoprecipitation experiments in NIH3T3 cells using anti-c-Jun. CSK was detected by Western blot and the results confirmed that CSK interacted with c-Jun in NIH3T3 cells (Fig. 2B).

To verify the in vitro experimental results, which showed that CSK could phosphorylate c-Jun at tyrosine sites, transient transfection experiments were used to test whether CSK could phosphorylate c-Jun in vivo. The pACT-csk and the pcDNA4-HismaxA vector or pcDNA4-HismaxA-c-jun were transiently transfected into HEK 293 cells. Forty hours after transfection, His-tagged proteins were purified by Ni-NTA magnetic beads, and protein expression was detected by Western blot using different antibodies. The results confirmed that CSK could bind with c-Jun and phosphorylate it at tyrosine sites in vivo (Fig. 2C).

**CSK phosphorylates c-Jun at Y26 and Y170.** After confirming that CSK phosphorylates c-Jun at tyrosine sites in vitro and in vivo, the next important step is to identify the tyrosine sites of c-Jun that are phosphorylated by CSK. To address this question, we first identified potential tyrosine sites using NetPhos 2.0 (15). c-Jun contains five tyrosine sites (Fig. 3A) and the results of NetPhos 2.0 analysis showed that Y170, Y26, and Y10 are three sites that might be phosphorylated by CSK. We designed six peptides based on a computer analysis (Fig. 3A) to determine the tyrosine phosphorylation sites of c-Jun. Because the Y26 and Y28 sites of c-Jun are close together, we designed three peptides to distinguish between these possible phosphorylation sites. The in vitro kinase assays using different c-Jun peptides as substrates for CSK were carried out in the presence of [γ<sup>32</sup>P]ATP. The results indicated that Y26 and Y170 were the two sites in c-Jun that were phosphorylated by CSK, whereas Y10, Y28, or Y190 in c-Jun cannot be phosphorylated by CSK (Fig. 3B). CSK is reported to be a highly selective tyrosine kinase in its phosphorylation of substrates (16). Therefore, we believed that our in vitro kinase assay results are reliable, but they still need to be confirmed using full-length c-Jun. We replaced each of the five tyrosine sites of c-Jun with phenylalanine and also made a double c-Jun mutant with Y26F and Y170F (YY26,170FF). The GST-mutant c-Jun proteins were expressed individually in E. coli BL21 at 30°C for 0.5 to 4 hours by the addition of 0.2 mmol/L isopropyl-β-D-galactopyranoside. The in vitro kinase reactions of active CSK with GST-c-Jun or GST-c-Jun-mutants as substrates were carried out in the presence of [γ<sup>32</sup>P]ATP. Samples were analyzed by SDS-PAGE and autoradiography. The results indicated that mutant Y170F or double mutant YY26,170FF c-Jun could not be phosphorylated by CSK, suggesting that Y170 of c-Jun is the most important site phosphorylated by CSK (Fig. 3C). The signal decreased slightly for the c-Jun mutant Y26F compared with GST-c-Jun wild-type (Fig. 3C), but the result was not clear enough to conclude whether CSK could phosphorylate Y26 of c-Jun. Thus, to determine whether Y26 of c-Jun could be phosphorylated by CSK, we used GST-c-Jun (1-89; Cell Signaling, Beverly, MA) as the substrate to repeat the in vitro kinase assay, and the result indicated that CSK could indeed phosphorylate c-Jun at this tyrosine site (Fig. 3D) in vitro. The Y26 site is the only possible c-Jun site that can be phosphorylated between c-Jun residues 1 and 89 by CSK. Considering the results of the peptide site mapping and the in vitro kinase assay with GST-c-Jun-mutants; we believed that Y26 of c-Jun can also be phosphorylated by CSK. However, for reasons as yet unclear, the phosphorylation of Y26 was weaker than the phosphorylation of Y170 by CSK in vivo.

**AP-1 activity and cell transformation are inhibited in CSK<sup>+/−</sup> but not in CSK<sup>−/−</sup> cells.** Subsequently, we determined the biological consequence of the phosphorylation of c-Jun by CSK. c-Jun is a signal-transducing component of the AP-1 transcription...
factor complex, which is very important for cell transformation. In addition, c-Jun itself has transforming activity when overexpressed in chicken embryo fibroblasts (17). Therefore, we tested whether the phosphorylation of c-Jun by CSK had an effect on cell transformation through its effects on AP-1 activity. We examined the role of CSK in cell transformation using CSK+/+ and CSK−/− (18) fibroblasts in the presence (10 ng/mL) or absence of EGF. Under normal growth conditions without EGF treatment, the CSK−/− cells generated more and bigger colonies, whereas CSK+/+ cells generated very few colonies (Fig. 4A and C). In addition, in untreated CSK−/− cells, AP-1 activity was much higher than that in CSK+/+ cells (Fig. 4B). These data strongly indicated that CSK−/− cells had a greater transforming ability and higher AP-1 activity. Combining these results with the finding that CSK phosphorylates c-Jun suggested that CSK had the ability to inhibit c-Jun function through its phosphorylation of c-Jun.

CSK, but not c-Src, regulates c-Jun total protein levels under normal culture conditions. In our experiments, we noticed that under normal cell culture conditions, c-Jun total protein level was higher (~1.7-fold) in CSK−/− cells compared with CSK+/+ cells. In addition, the phosphorylation of c-Jun at Ser63 and Ser73 was fairly low. On the other hand, phosphorylation of Src at Tyr528, detected at the same time as c-Jun on the same membrane, was much stronger in CSK+/+ cells compared with CSK−/− cells (Fig. 5A). In cells treated with EGF, the total c-Jun level was also higher in CSK−/− cells than in CSK+/+ cells, but the basal or EGF-induced phosphorylation of c-Jun at Ser63 and Ser73 in CSK−/− cells showed no dramatic difference between cell lines. CSK was reported to negatively regulate Src function by phosphorylating Src at Tyr528 (19). Thus, the higher level of total c-Jun might be caused by the higher activity of Src in CSK−/− cells because of the absence of the inhibitory function of CSK. Thus, we examined whether Src had an effect on the total c-Jun protein level and phosphorylation at Ser63 and Ser73 of c-Jun in Src-deficient SYF cells and SYF cells overexpressing c-Src (20). The results indicated that the total levels of c-Jun under normal culture conditions were not different between the two cell lines and the phosphorylation of c-Jun at Ser63 and Ser73 without stimulation was barely detectable. Following treatment with EGF, the total level of c-Jun and the phosphorylation of c-Jun at Ser63 and Ser73 was slightly higher in SYF cells expressing c-Src than in SYF (−Src) cells (Fig. 5B). This suggested that c-Src might be involved in higher levels of c-Jun expression stimulated by EGF treatment but not under normal culture conditions without added EGF. Furthermore, EGF strongly induced c-Jun phosphorylation at Ser63 and Ser73 in CSK−/− cells or SYF (−Src) cells, indicating that phosphorylation of c-Jun at Ser63 and

---

**Figure 3.** Mapping of the c-Jun tyrosine site(s) phosphorylated by CSK. A, c-Jun phospho-tyrosine sites predicted by NetPhos 2.0 and peptide design following prediction. WT, wild-type; Mu, mutant; Score, possibility that the site might be phosphorylated in vivo; Pred, prediction. B, peptide mapping of the tyrosine sites phosphorylated by CSK as determined by an in vitro kinase assay in the presence of 32P as visualized by autoradiography. C, confirmation of the peptide mapping results by using GST-c-Jun or a GST-c-Jun mutant as substrate in the in vitro kinase assay in the presence of 32P as visualized by autoradiography. D, confirmation of Y26 in c-Jun as a phosphorylation site of CSK using GST-c-Jun (1-89) as a substrate in an in vitro kinase assay in the presence of 32P as visualized by autoradiography or by Western blotting.
Ser73 occurred independently of CSK or Src kinase activity. The level of phosphorylated tyrosines of c-Jun under normal cell culture conditions was similar in SYF (-Src) cells and SYF (+Src) cells (Fig. 5C). These results suggested that Src was neither related to the total c-Jun protein level nor to the level of phosphorylated tyrosines of c-Jun under normal cell culture conditions.

Under normal culture conditions, the phosphorylation level of c-Jun at Ser63 and Ser73 in either CSK+/+ or CSK−/− cells was barely detectable and therefore, presumably, the activity of JNKs or ERKs, which phosphorylate c-Jun at Ser63 and Ser73, was also weak. Under EGF stimulation conditions, CSK activity is inhibited (21) and ERK or JNK is activated, and c-Jun is phosphorylated at Ser63 and Ser73. The mechanism of c-Jun regulation by ERKs or JNKs under EGF stimulation is well-characterized and CSK activity is inhibited with EGF stimulation. Therefore, in our experiments, we focused only on the regulation of c-Jun under normal culture conditions.

**CSK-mediated phosphorylation promotes c-Jun degradation.** The ubiquitin-dependent proteolytic pathway plays an important role in protein degradation, and the activity of many short-lived regulatory proteins comprising numerous transcription factors is controlled by proteolysis through the ubiquitin pathway. The phosphorylation-dependent ubiquitination of c-Jun plays an important role in its function (11, 22, 23). Based on our results thus far, c-Jun may likely be targeted for degradation after phosphorylation by CSK. To test this hypothesis, GST wild-type c-Jun and the GST c-Jun double mutant (c-Jun-YY26,170FF) proteins were used for an in vitro ubiquitination assay. Results (Fig. 6A) indicated that GST wild-type c-Jun had a higher level of ubiquitination compared with the GST c-Jun double mutant (c-Jun-YY26,170FF). To further confirm this result, c-Jun was immunoprecipitated from CSK+/+ cells and CSK−/− cells and used for Western blot analysis to detect c-Jun, ubiquitin, and phosphorylation of tyrosines. Results indicated a much higher level of phosphorylated tyrosines (3.3-fold) and a lower level of total c-Jun (∼0.7-fold) protein present in CSK+/+ cells compared with CSK−/− cells (Fig. 6B). In addition, ubiquitination of c-Jun was greater in CSK+/+ cells and loss of functional CSK inhibited c-Jun ubiquitination (Fig. 6B). One possible reason for the persistent tyrosine phosphorylation signal for c-Jun in CSK−/− cells is most likely due to the presence of other c-Jun tyrosine kinases, such as c-Abl, in these cells (9). These results suggested that CSK-mediated c-Jun phosphorylation promotes ubiquitin-dependent degradation of c-Jun. Next, the stability of c-Jun was analyzed in CSK+/+ cells and CSK−/− cells by immunoblotting after inhibition of protein synthesis with cycloheximide. The c-Jun protein was much more stable in CSK−/− cells than in CSK+/+ cells (Fig. 6C). A pulse-chase experiment was done to determine the half-life of c-Jun in CSK+/+ cells and CSK−/− cells and the results indicated that c-Jun had a longer half-life in CSK−/− cells than in CSK+/+ cells (Fig. 6D). Overall, our results indicated that c-Jun is phosphorylated by CSK in CSK+/+ cells under normal growth conditions and is more likely to be degraded through the ubiquitination pathway (Fig. 6), resulting in a lower total level of c-Jun protein in CSK+/+ cells (Figs. 5A and 6). In contrast, c-Jun cannot be phosphorylated by CSK in CSK−/− cells and is thus less likely to be degraded through the ubiquitination pathway, resulting in a higher total level of c-Jun in these cells. The decreased c-Jun protein level associated with CSK phosphorylation is related to c-Jun stability, and therefore, is also related to AP-1 activity and cell transformation. Our data therefore suggested that a lower phosphorylation level of c-Jun by CSK is responsible for higher AP-1 activity and cell transformation in CSK−/− cells. However, stimulation with EGF can down-regulate the CSK protein and its subsequent activity (21), resulting in increased AP-1 activation and cell transformation caused by c-Jun.
Discussion

CSK is a protein tyrosine kinase which selectively phosphorylates Src and negatively regulates Src activity (18, 19, 24). CSK negatively regulates cell proliferation during development in Drosophila by inhibiting Src activity (25). It also negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun NH2-terminal kinase, and STAT pathways (26). Reduced CSK activities have been found in hepatocellular carcinoma (27) and in colorectal carcinoma (28). Overexpression of the csk gene suppresses tumor metastasis (29, 30) and CSK also inhibits transcriptional activation of AP-1 (13). CSK inhibits cell transformation through negative regulation of Src activity. In this report, we found that under normal culture conditions, CSK inhibits AP-1 activity and cell transformation caused directly by c-Jun. This function of CSK is not dependent on the inhibition of Src kinase.

We found that CSK phosphorylates c-Jun directly. The phosphorylation of c-Jun by CSK promotes ubiquitin-dependent degradation of c-Jun, thereby inhibiting AP-1 activity and cell transformation independent of Src inhibition. Our data can be summarized in the model in Fig. 6E. Under normal growth conditions, CSK phosphorylates c-Jun at Y26 and Y170, and the phosphorylated c-Jun is more likely to be degraded through the ubiquitination pathway. On the other hand, JNKs, ERKs, or Src are not activated and therefore c-Jun is not phosphorylated at Ser63 or Ser73. AP-1 activity decreases because of this negative regulation of c-Jun by CSK. By this mechanism, c-Jun is degraded and maintained at a low steady-state level resulting in low AP-1 activity and proper control of cell proliferation under normal growth conditions. Conversely, under stimulation conditions, CSK activity is inhibited (21) and the phosphorylation of c-Jun at Y26 and Y170 is also suppressed. This results in a decrease in the degradation of c-Jun and an increase in total c-Jun protein level. At the same time, JNKs or ERKs are activated and the phosphorylation of c-Jun at Ser63 and Ser73 increases. c-Jun is more stable, total c-Jun protein level increases, and AP-1 activity is higher (Fig. 6E).

The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin system. Ubiquitin-mediated degradation of regulatory proteins plays an important role in the control of numerous processes (31). The liability of signal-dependent transcription factors is crucially important because changes in their stabilities could have a significant effect on activities of downstream target genes. c-Jun is activated after phosphorylation at Ser63 and Ser73 by mitogen-activated protein kinases (MAPK). The activation of c-Jun after phosphorylation is accompanied by a reduction in c-Jun ubiquitination (11). The δ domain of c-Jun is also suggested to mediate the ubiquitin-dependent c-Jun degradation (22). MAPKs inhibit c-Jun ubiquitination after exposure of cells to growth factors or stress. However, under normal growth conditions, CSK is responsible for c-Jun ubiquitin–dependent degradation and is more important in regulating c-Jun stability, although MAPKs are not activated. The function of CSK is suppressed after exposure of cells to growth factors or stress, and the MAPKs are activated and are responsible for c-Jun stability. Thus, CSK and MAPK act in opposition to

Figure 5. CSK, but not c-Src, is associated with c-Jun total protein levels under normal culture conditions. A, total level of c-Jun under normal cell culture conditions is higher in CSK−/− cells than in CSK+/+ cells with or without EGF stimulation. No dramatic difference was observed in c-Jun (Ser63 and Ser73) phosphorylation in wild-type or knockout CSK cells. B, total level of c-Jun expression under normal cell culture conditions is similar in SYF (−Src) cells and SYF (+Src) cells with or without EGF stimulation. c-Jun phosphorylation at Ser63 or Ser73 in SYF (−Src) cells is slightly weaker than in SYF (+Src) cells. C, the level of phosphorylated tyrosine of c-Jun under normal cell culture conditions is similar in SYF (−Src) cells and SYF (+Src) cells.
regulate c-Jun ubiquitin–dependent degradation under different circumstances, and by this mechanism, CSK and MAPKs facilitate cellular responses to environmental changes.

c-Jun is suggested to contain an activator domain (A1) that is negatively regulated by a cell type–specific inhibitor (32). The HDAC3-containing repressor complex was identified (33) and a de-repression model, which regulates c-Jun transcription activity, was provided (34). The Y26 and Y170 phosphorylation sites are located in the A1 activation domain in c-Jun. c-Jun has been reported to be phosphorylated at Y170 by c-Abl, and this process is inhibited when JNK is activated (9). Our data strongly suggested that CSK is one of the repressors that can bind with c-Jun at the A1 activation domain, and negatively regulate c-Jun function through ubiquitin-dependent degradation, thereby regulating AP-1 activity. The repression caused by CSK can be relieved by stimulation, such as EGF treatment. A low level of CSK has been observed in carcinogenesis, and our model suggests a new mechanism by which cell transformation can be caused by loss of functional CSK. The loss of CSK results in an increased AP-1 activity by allowing c-Jun to escape the ubiquitin-dependent degradation that would normally follow its phosphorylation by CSK.

Acknowledgments

Received 12/16/2005; revised 2/21/2006; accepted 3/22/2006.

Grant support: The Hormel Foundation and NIH grants CA77646, CA88961, CA111536, CA111356, and CA27502.

Figure 6. CSK-mediated c-Jun phosphorylation promotes c-Jun degradation. A, in vitro ubiquitination assay for GST wild-type c-Jun or GST c-Jun double mutant (YY26,170FF). B, the level of phosphorylated tyrosine was much higher in CSK+/+ cells than in CSK−/− cells and the total amount of c-Jun protein was also higher in CSK−/− cells than in CSK+/+ cells. Ubiquitination of c-Jun was identified in CSK−/− cells or CSK+/+ cells at the indicated time points by immunoblotting after addition of cycloheximide. D, stability of c-Jun in CSK−/− cells or CSK+/+ cells was analyzed by pulse-chase experiments. E, schematic illustration of the regulation of c-Jun ubiquitination. Under normal growth conditions, CSK phosphorylates c-Jun, which promotes ubiquitin-dependent degradation of c-Jun, resulting in a decrease in AP-1 activity. In contrast, with EGF stimulation, CSK is inhibited and c-Jun is activated by phosphorylation by MAP kinases, which inhibits ubiquitin-dependent degradation of c-Jun, resulting in an increased AP-1 activity.
References
COOH-Terminal Src Kinase–Mediated c-Jun Phosphorylation Promotes c-Jun Degradation and Inhibits Cell Transformation

Feng Zhu, Bu Young Choi, Wei-Ya Ma, et al.