Identification of c-Fos as a mitotic phosphoprotein: regulation of c-Fos by Aurora-A

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Abstract

The c-Fos has been implicated in the regulation of gene expression under a variety of stimuli. It is known that c-Fos undergoes protein phosphorylation, which may subsequently modulate diverse functions in cells. However, less is known about the role and phosphorylation status of c-Fos during mitosis. Here, we showed that c-Fos exhibited an electrophoretic mobility up-shift as detected by SDS-PAGE during mitosis, which is an indication of protein phosphorylation. Aurora-A, but not Aurora-B or -C, serves as one of the kinases catalyzing the mitotic phosphorylation of c-Fos. The mobility up-shift was partially abolished by introducing siRNA or a catalytically inactive form of Aurora-A. Moreover, ectopic expression of the wild type, but not the catalytically inactive form of Aurora-A resulted in the alteration of c-Fos complex formation, suggesting Aurora-A is engaged in the regulation of c-Fos protein–protein interaction. These findings imply that c-Fos may undergo cell cycle dependent phosphorylation, in which some kinases including Aurora-A play a role in catalyzing the post translational modification of c-Fos.

Introduction

The c-Fos acts as an immediate early gene responding to various external stimuli [1–4]. Its induction may be both very rapid and significant [5, 6]. The c-Fos protein contains a basic region mediating sequence-specific DNA-binding and a leucine zipper, which is required for dimerization of the c-Fos and Jun family [7]. By forming a heterodimer with members of the Jun family [7], c-Fos acts as a transcription factor referred to as activator protein-1 (AP-1) [8] which can stimulate target gene expression by binding to an AP-1 site [9]. The c-Fos proto-oncoprotein is a key cell regulator, which may transform cells [10] and is therefore involved in tumorigenesis [11, 12]. Several kinases have been documented to phosphorylate c-Fos, including the MAP kinase (MAPK) family [13–15], protein kinase A (PKA) [16] and ribosomal S6 kinase (RSK) [15]. In general, MAPK and PKA are versatile kinases and play a range of important roles. Interestingly, these two kinases have been documented as participating in the regulation of mitosis [17–19] and this accordingly raises the possibility that c-Fos may undergo mitotic phosphorylation in cells even though RSK is inactivated in mitosis [20].

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Aurora-A, a mitotic serine/threonine kinase, has attracted intense attention because of its position as a potential oncogene in a variety of human cancers [21]. To understand how Aurora-A works in cells, it is imperative to screen its downstream substrates. By systematic analysis [22, 23] and individual studies, many substrates of Aurora-A have been identified and these include transcription factors and transcription regulatory factors such as p53 [24] and BRCA1 [25]. As a mitotic kinase with many cellular activities, Aurora-A has been shown to be engaged in the regulation of spindle assembly [26], centrosome maturation [27], the mitotic checkpoint [28], cell cycle progression [29], oocyte maturation [30] and embryonic development [31].

In the studies we have identified c-Fos as a mitotic phosphoprotein and showed that Aurora-A is one of the kinases catalyzing c-Fos phosphorylation during mitosis. The observation that overexpression of functional or catalytically inactive mutant of Aurora-A disturbed the complex formation of c-Fos may suggest a role of the Aurora-A-dependent phosphorylation in modulating protein–protein interaction of c-Fos. These studies represent the first effort focusing on an exploration of c-Fos phosphorylation during mitosis, and thus may not only help to delineate c-Fos regulation along the cell cycle, but should also lead to further investigations that explore the potential functional link between c-Fos and Aurora-A during a diverse range of biological responses.

**Materials and methods**

**Materials**

All restriction enzymes were purchased from New England BioLabs. Fetal bovine serum (FBS), Dulbecco’s modified eagle medium (DMEM), penicillin, streptomycin, and Lipofectamine™ were purchased from GIBCO-BRL. The [35S]-methionine was from Perkin-Elmer Life Sciences.

**Cell cultures, transfection and synchronization**

HeLa cells and 293T cells were purchased from the American Type Culture Collection (ATCC), maintained in humidified incubators at 37°C in the presence of 5% CO₂ and were grown in DMEM medium containing 10% FBS, 100 unit/ml penicillin and 100 μg/ml streptomycin. Transfection of cells was performed with Lipofectamine™ according to the manufacturer’s instructions. Cells were synchronized at G1/S boundary by thymidine and aphidicolin. Briefly, HeLa cells were cultured for 14 h in the presence of 2 mM thymidine (Sigma), released for 12 h in fresh medium, and arrested for 14 h in the presence of 1.6 mg/ml aphidicolin (Sigma). Subsequently, cells were released from the aphidicolin block by changing to fresh culture medium in the absence of aphidicolin. The percentage of cells at mitosis, i.e., mitotic index, was measured by morphological observation of DNA configuration via DAPI staining. Alternatively, to enrich mitotic cells, 50 ng/ml of nocodazole was added for 16 h and then cells were harvested for Western blot.

**Preparation of recombinant proteins, in vitro transcription/translation, in vitro kinase reaction and dephosphorylation assay**

Human Aurora-A and -B cDNAs were subcloned into the vector pGEX4T and expressed in E. coli as GST-fusion protein as described earlier [22]. The proteins were purified with glutathione beads. Aurora-C was expressed and purified as described earlier [32]. The c-Fos cDNA constructed in pCDNA3.1 with T7 promoter was served as templates for in vitro transcription and translation (TNT Quick Coupled Transcription/Translation Systems from Promega) in the presence of [35S]-methionine. The procedures were according to the manufacture’s instruction except that we added 0.5 μg of plasmid per reaction and the reaction volume was scaled down to 20 μl. The [35S]-methionine labeled c-Fos was incubated with recombinant protein of each member of Aurora family in the kinase reaction buffer (25 mM Tris–HCl, pH 7.4, 10 mM MgCl₂, 100 μM ATP, 2 mM EGTA, 1 mM dithiothreitol, 1 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol) at 30°C for 30 min. The reaction volumes were 40 μl. To perform the protein dephosphorylation experiment, lysates or isotope-labeled TNT products were incubated with 400 units of lambda phosphatase (New England Biolabs) in lambda phosphatase reaction buffer (50 mM Tris, pH 7.8, 2 mM MnCl₂, and 5 mM dithiothreitol) at 30°C for 30 min.