STAT3 Ser-727 Phosphorylation by CDK1 Inhibits STAT3 Function during Cell Cycle Progression

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Abstract

Signal Transducer and Activator of Transcription 3 (STAT3) mediates cellular responses to diverse cytokines and growth factors by modulating the expression of specific target genes. While phosphorylation of STAT3 at Tyr-705 has been demonstrated to be a prerequisite for STAT3 dimerization, nuclear translocation and activation of gene transcription, the role of Ser-727 in regulation of STAT3 activity is controversial. Using Kinetworks™ KPSS 1.1 phospho-site immunoblotting screening of nocodazole-treated HeLa cells, STAT3 Ser-727 phosphorylation was enhanced during mitosis, and this correlated with a reduction of Tyr-705 phosphorylation. Over-expression of STAT3 mutants in which these phosphorylation sites were separately abolished revealed that phosphorylation at these sites appeared to be mutually antagonistic. The nocodazole-induced STAT3 Ser-727 phosphorylation was reduced by selective inhibition of cyclin-dependent kinase 1 (CDK1) phosphotransferase activity, and CDK1 could directly phosphorylate GST-STAT3 Ser-727 *in vitro* and it co-immunoprecipitated with STAT3 from cell lysates. Blocking Ser-727 phosphorylation through transfection of HEK-293 cells with its nonphosphorylatable mutant enhanced STAT3 DNA binding activity towards its target gene promoters, implying a negative effect of Ser-727 phosphorylation on its transcriptional activity. Interference of Ser-727 phosphorylation resulted in an exit from mitotic arrest induced by nocodazole treatment and a cell cycle arrest at G1 phase, as indicated by the accumulation of 2N cell population and enhanced expression of G1 cell cycle regulators including $p21^{CIP/WAF1}$, $p27^{Kip1}$ and cyclin E. Our observations point to a novel role of STAT3 Ser-727 phosphorylation in control of the onset and maintenance of M-phase during the cell cycle through down-regulation of CDK inhibitors.

Introduction

STAT3 belongs to a family of signal transducing transcription factors that play critical roles in mediating cellular responses to diverse cytokines and growth factors by modulating the expression of specific target genes. Engagement of cytokines and growth factors to their cognate receptors induces STAT3 Tyr-705 (Y705) phosphorylation either by receptor-associated tyrosine kinases such as in the Janus kinase (JAK) and Src kinase families or by intrinsic receptor-tyrosine kinases such as the epidermal growth factor receptor (EGFR). This leads to STAT3 dimerization through reciprocal phospho-tyrosine-SH2 domain interactions, nuclear translocation and binding to enhancer/promotor sequences of target genes.

Constitutive activation of STAT3 is found in a wide variety of malignancies including cancers, and the mechanisms underlying the activation of STATs have been the focus of extensive studies in recent years. While the tyrosine phosphorylation of STATs mediated by nonreceptor or receptor tyrosine kinases seems to be the paradigm for STAT activation utilized by a large number of cytokines and growth factors, there is also the phosphorylation of a serine residue within the COOH-terminal transcriptional activation domain of most STATs. The serine residue is located within a conserved PMSP (or PSP) motif among STAT1, 3, 4 and 5, but not STAT2 and 6, and corresponds to Ser-727 (S727) in STAT3. It has been demonstrated that mitogen-activated protein kinases (MAPKs), protein kinase C-delta isoform (PKCδ), the mammalian target of rapamycin kinase (mTOR) and protein kinase R (PKR) mediate serine phosphorylation either directly or indirectly in a stimulus- or cell type-dependent manner, indicating that STATs may be convergent points for integrating signals from multiple pathways. The effect of this serine phosphorylation on STAT DNA-binding/transcriptional activity has been controversial.

During experiments in which Kinetworks™ KPSS phospho-site profiling was performed on nocodazole treated HeLa cells blocked in M phase, a marked enhancement of STAT3 S727 phosphorylation was observed. The objective of this study was to determine how this was achieved and the physiological role for this event..

Materials and Methods

Cell Lines and Culture Conditions - HeLa (human cervical carcinoma cells) and HEK293 (human embryonic kidney cells) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), in a humidified atmosphere containing 5% CO_2. The culture dishes used for HEK293 cells were treated with 2% poly-L-lysine for 20 min prior to each use.

Plasmids - pGEX plasmids containing glutathione-S-transferase (GST)-STAT3 wild-type (WT) and STAT3 point mutations at S727 (replaced by A, S727A) or Y705 (replaced by F, Y705F) were constructed. STAT3 expression plasmids for mammalian cell transfection experiments were prepared by inserting the *EcoRI-XhoI* fragments containing amino acids 28-770 of STAT3 from GST-STAT3 WT or mutants into pcDNA3. Flag sequences were incorporated into their C-terminii as tags.

Transient Transfection - At 40-60% confluency, HEK293 cells were transiently transfected with plasmids as indicated using Lipofectamine transfection reagent.

Electrophoretic mobility shift assays - Twenty μg of total HEK293 cell lysates were used in each assay carried out using STAT3 gel shift oligonucleotide pre-labeled with [γ^{32}P]ATP. The resulting protein/DNA complexes were immunoprecipitated using anti-Flag antibody, and then resolved on a 13% native polyacrylamide gel prior to detection with a fluorescence imaging analyzer.

Kinetworks™ Proteomics Analysis - Total cell lysates were prepared by sonication for 15 sec in lysis buffer [pH 7.4, 20 mM Tris, 20 mM β-glycerophosphate, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na_2VO_4, 0.5% Nonidet P-40, and 1 mM dithiothreitol] supplemented with protease inhibitors [1 mM phenylmethanesulfonylfluoride (PMSF), 2 μg/ml leupeptin, 4 μg/ml aprotinin and 1 μg/ml pepstatin A] and centrifugation at 100,000 rpm for 30 min at 4°C. Protein concentration was determined by the Bradford assay. The Kinetworks™ KPSS 1.1 phospho-site analysis was performed with 300 μg of total protein (protocols available at www.kinexus.ca).

Flow cytometry - Twenty-four hours after transfection with STAT3 WT or S727A, HEK293 cell cultures were re-plated into new dishes to prevent overconfluence. Nocodazole treatment was administrated at 24 h after re-plating for 20 h. Following trypsinization, cells were fixed in cold methanol and stained with propidium iodide in the presence of RNase A. The DNA fluorescence was measured using a FACScan; data acquisition and analysis were performed with the CellQuest software from Becton Dickinson.

BrdU incorporation assays and immunofluorescence microscopy — STAT3 WT and S727A-transfected HEK293 cells were grown on coverslips and treated with nocodazole for 20 h. Following removal of the nocodazole, the cells were then incubated with 20 μM BrdU for 6 h. Following fixation and permeabilization, the cells were stained with anti-BrdU and anti-Flag antibodies for 2 h at room temperature. Their respective signals were detected by Alexa488-labeled anti-mouse IgG and Alexa569-labeled anti-rabbit IgG secondary antibodies for 1 h in the dark. Nuclei were counter-stained with propidium iodide following RNaseA digestion.

Results

STAT3 S727 phosphorylation is an mitosis-associated event - Exposure of proliferating HeLa cells to 200 ng/ml nocodazole for 20 h induced a cell-cycle arrest in mitosis and this was accompanied by changes of a wide range of cell signaling proteins in their phosphorylation states, as revealed by the Kinetworks™ KPSS 1.1 phosphoprotein screen (Fig. 1). This screen utilizes phospho-site-specific antibodies to systematically track changes in phosphorylation states of 30 known signaling proteins. Unexpectedly, the phosphorylation of S727 in STAT3 (Band 35) was enhanced by about 3.5-fold after 20 h incubation with nocodazole. Treatment of HeLa with other microtubule-interfering agents such as taxol, vinblastine, and colchicine at concentrations effective in causing the mitotic arrest also led to the increase of STAT3 S727 phosphorylation to different degrees (Fig.2A). Furthermore, increased phosphorylation of STAT3 S727 phosphorylation occurred during normal mitosis in HeLa cells synchronized by releasing serum-starved cells from G_0/G_1 block with serum addition (Fig. 2C).

KPSS 1.1 Phospho-sites tracked:

1. Adducin α - S724
2. Adducin γ - S693
3. B23 - S4
4. CDK1 - Y15
5. CREB - S133
6. ERK1 - T202+Y204
7. ERK2 - T185+Y187
8. GSK3α - S21
9. GSK3α - Y279
10. GSK3β - S9
11. GSK3β - Y216
12. JNK - T183+Y185
13. cJun - S73
14. MEK1 - S217+S221
15. MEK3/6 - S189/S207
16. MSK1 - S376
17. NR1 - S896
18. p38α - T180+Y182
19. PKBα - T308
20. PKBα - S473
21. PKCα - T638δ.
22. PKCα - S657
23. PKCδ - T505
24. PKCε - S719
25. PKR - T451
26. Raf1 - S259
27. RB1 - S780
28. RB1 - S809+S811
29. RSK1 - T360+S364
30. S6K p70 - T389
31. Smad1 - S463+S465
32. Src - Y418
33. Src - Y529
34. STAT1 - Y701
35. STAT3 - S727
36. STAT5 - S694

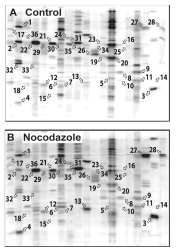

Figure 1. Kinetworks™ KPSS-1.1 phospho-site profile of HeLa cells in response to 200 ng/ml nocodazole treatment for 20 h.

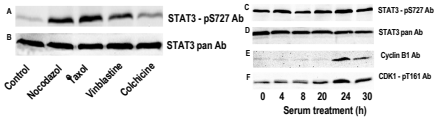

Figure 2. Phosphorylation of STAT3 S727 is associated with mitosis.

Reciprocal inhibition between S727 and Y705 phosphorylation - Concomitant to the increase of STAT3 S727 phosphorylation with treatment of HeLa cells with nocodazole for 20 h, there was a reduction of STAT3 phosphorylation at Y705 (Fig. 3). We examined the two phosphorylation events in HEK293 cells expressing wild-type (WT) STAT3 or non-phosphorylatable mutants, S727A or Y705F. Upon 20 h nocodazole treatment, whereas the over-expression of STAT3 S727A mutant abrogated S727 phosphorylation as expected, a higher level of S727 phosphorylation was observed in STAT3 Y705F-expressing cells as compared to STAT3 WT-transfected cells (Fig.3D). Conversely, Y705 phosphorylation was further enhanced in S727A-expressing cells relative to that in WT-transfected cells.

CDK1 phosphorylates STAT3 at S727 - Several protein-serine/threonine kinase inhibitors were employed along with nocodazole to examine their effects on STAT3 S275 phosphorylation; these included the Mek1/2 inhibitor PD98059, the p38 MAPK inhibitor SB203580, the JNK1/2 inhibitor SP600125, the CK2 inhibitor DRB (5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole), the PI 3-kinase inhibitor LY294002, the CDK1/2 inhibitor olomoucine, the mTOR inhibitor rapamycin, and the PKC inhibitor RO318220. Among them, only olomoucine perturbed nocodazole-induced S727 phosphorylation significantly, while no effects were observed on STAT3 protein expression, indicating the possible involvement of CDK1 in the pathway leading to S727 phosphorylation (Fig. 4 left). Similar inhibitory effect on STAT3 S727 phosphorylation was also observed in Roscovitine, another potent and selective inhibitor of CDKs (Fig. 4 right).

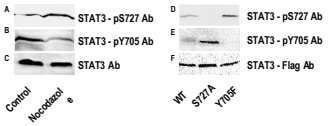

Figure 3. Reciprocal inhibition between S727 and Y705 phosphorylation in STAT3.

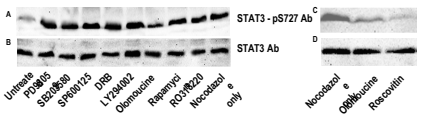

Figure 4. CDK1 mediates nocodazole-induced STAT3 S727 phosphorylation.

To confirm the role of CDK1 in STAT3 S727 phosphorylation, we tested whether STAT3 could be phosphorylated at S727 by CDK1 *in vitro*. Purified active recombinant CDK1/cyclin B1 complex from transfected insect cells phosphorylated GST-STAT3 at S727 (Fig. 5).

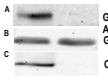

Figure 5. CDK1 is a direct STAT3 S727 kinase *in vitro*.

To further explore the possible regulation of STAT3 S727 phosphorylation by CDK1, we next investigated whether CDK1 directly interacted with STAT3 *in vivo*. Reciprocal immunoprecipitations with either CDK1 or Flag antibody were carried out in lysates of HEK293 cells transfected with either a Flag-tagged STAT3 WT or a S727A mutant construct, followed by nocodazole treatment for 20 h (Fig. 6). The strongest interaction was found between CDK1 and STAT3 S727A, consistent with the kinetic relationship between an enzyme and a pseudo substrate.

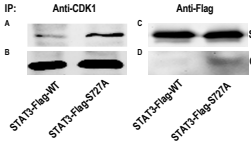

Figure 6. CDK1 and STAT3 form complexes *in vivo*.

STAT3 S727 phosphorylation by CDK1 attenuates its DNA-binding activity - We compared the DNA-binding activities of STAT3 WT and STAT3 S727A over-expressed in HEK293 towards a double-stranded oligonucleotide containing the consensus binding site for STAT3 (5'-GAT CCT TCT GGG AAT TCC TAG ATC-3') in electrophoretic mobility shift assays. As shown in Figure 7, while both proteins were expressed at similar levels, more STAT3 S727A proteins were found in complexes with DNA in the STAT3 immunoprecipitates than STAT3 WT, indicating that STAT3 S727A possesses stronger DNA binding activity when compared to its wild-type counterpart.

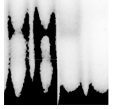

Figure 7. Inhibition of S727 phosphorylation enhances STAT3 DNA-binding activity.

Inhibition of S727 phosphorylation abolishes nocodazole-induced mitotic arrest - Cell cycle analysis using flow cytometry revealed that about 40% of the cells population transfected with STAT3 WT accumulated in a tetraploid (4N) state upon a 20-h nocodazole treatment (Fig.8). Examination of cell morphology as well as chromosome configuration indicated that most cells were in mitotic arrest induced by nocodazole as expected. In sharp contrast, transfecting STAT3 S727A resulted in the accumulation of around 70% of the cells in 2N DNA states while only less than 5% of the cells remained as 4N, indicative of a cell cycle arrest at G1 phase (Fig.8). The decrease of 4N cells coupled with the increase of 2N cells implies that the mitotic arrest induced by nocodazole was lost and the cells underwent a mitotic exit and proceeded into and remained in the next G1 phase.

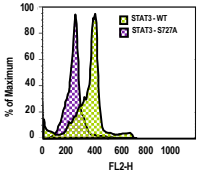

Figure 8. Inhibition of S727 phosphorylation results in loss of mitotic arrest induced by nocodazole and a G1 arrest in the next cell cycle.

To further examine the molecular mechanism underlying the role of S727 phosphorylation in nocodazole-induced mitotic arrest, we evaluated the effects of STAT3 S727A transfection on the expression of 30 cell cycle-related proteins using Kinetworks™ KCCP-1.0 cell cycle protein analysis (Fig. 9). In particular, in the STAT3 S727A transfected HEK293 cells, the expression levels of CDK4, CDK5, NEK2, 14-3-3 and p27 CDK inhibitor were elevated greater than 1.6-fold, and the expression levels of Cdc34, CDK7, and cyclin G1 were reduced by more than 50% (Fig. 9B). The expression-related changes of the these and other cell cycle proteins (not shown) also point to the abrogation of nocodazole-induced mitotic arrest upon the inhibition of S727 phosphorylation.

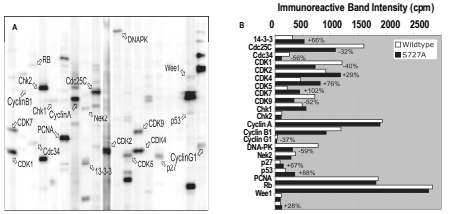

Figure 9. Kinetworks™ KCCP 1.0 cell cycle screen analysis of the effects of STAT3 S727 phosphorylation in HEK-239 cells.

If the STAT3 S727A-expressing cells were arrested in G1 phase as indicated above, then they should enter S-phase (DNA synthesis) sooner than their wild-type counterparts when they are released from nocodazole treatment. Over 60% of STAT3 S727A cells exhibited the BrdU incorporation at 6 h after nocodazole removal. In contrast, less than 15% of STAT3 WT cells were BrdU-positive (data not shown).

Conclusions

1. CDK1 directly phosphorylates STAT3 S727 during mitosis to inactive this transcription factor until critical mitotic events are completed.
2. The loss of phosphorylation of STAT3 S727 permits the activation of STAT3 function to facilitate cell division and release from drug induced M phase arrest.
3. Kinetworks™ analyses of nocadazole-arrested cells has revealed a multitude of phosphorylation changes in known target phosphoproteins and cross-reactive phosphoproteins. We recently identified one of these proteins as nucleophosmin/B23, and demonstrated that polo-like kinase 3 phosphorylation of serine-4 of B23 was critical for centromere duplication. We plan to continue to identify additional phosphoproteins that are affected during mitosis to establish their interconnection in signalling pathways and their functional roles in orchestrating the myriad of events that either facilitate the successful completion of mitosis or the initiation of apoptosis.