Polo-like kinase 1 Phosphorylation of B23/Nucleophosmin at Ser-4 in Control of Centrosome Duplication

Hong Zhang[1], Xiaoqing Shi[2], Harry Paddon[2] and Steven Pelech[1,2]

[1]Kinexus Bioinformatics Corporation, Vancouver, B.C. Canada;
[2]Dept. Medicine, University of British Columbia, Vancouver, B.C. Canada

Abstract

Kinexus has developed its Kinetworks™ proteomics services with highly validated antibodies to provide precise and reliable data about the expression levels and phosphorylation states of hundreds of signalling proteins in tissue and cell lysates. Microtubule-interfering agents such as nocodazole, taxol and vinblastine have been at the forefront of cancer chemotherapy due to the fundamental role that microtubules play in chromosomal segregation during mitosis. Kinetworks™ KPSS Phospho-site analyses have revealed marked changes in the phosphorylation of more than 120 target proteins proteins in various human tumour cell lines with nocodazole treatments. In addition, there were also profound changes in the phosphorylation of many other cross-reactive proteins, including a 40 kDa protein that was identified by mass spectrometry as B23 or nucleophosmin. B23 underwent enhanced phosphorylation at a novel site that was identified as Ser-4, which was confirmed by site-directed mutagenesis. Ser-4 phosphorylation of B23 by nocodazole treatment was dependent on the activation of the cyclin-dependent protein kinase CDK1, since it was abolished in cells that were also incubated with the CDK1 inhibitor olomoucine. Furthermore, the polo-like kinase Plk1 was shown to directly phosphorylate B23 Ser-4 phosphorylation in vitro, and this protein kinase is activated downstream of CDK1 during the cell cycle. Transfection of HeLa cells with recombinant B23 with Ser-4 mutated to Glu resulted in increased numbers of centrosomes, whereas the Ala-4 mutant form of B23 produced cells that had only one or no observable centrosomes. Our findings indicate that phosphorylation of Ser-4 of B23 may play an important role in the regulation of centrosome duplication, and point to Plk1 as an attractive target for the development of chemotherapy drugs to

Introduction

With the completion of the sequencing of the human genome, the most challenging and fruitful biomedical research endeavor of this decade will be the mapping of cell signalling systems and establishing their linkages to normal and disease related processes. Over 500 human protein kinases are responsible for the phosphorylation of as many as 100,000 sites in about 10,000 different proteins. Our knowledge of the architecture of protein kinase signalling networks is extremely rudimentary, but the application of systems biology approaches promises to uncover the true complexity of these networks. To this end, Kinexus has tested more than 1800 commercial antibodies for the accurate and quantitative detection of protein kinases and other signalling proteins in our Kinetworks™ immunoblotting services. With this methodology, we have previously reported novel interactions amongst protein kinases, including the binding of phosphorylated and activated p38 MAP kinase to the ERK1/ERK2 MAP kinases to prevent their phosphorylation and activation by MEK1 and MEK2 [Zhang et al. (2001) J. Biol. Chem. 276:6905]. We also observed the direct binding of p38 MAP kinase to casein kinase 2, and demonstrated that this mediated the phosphory-lation of the tumour suppressor protein p53 at Ser-392 to promote its activation [Sayed et al. (2000) J. Biol. Chem. 275:16569; Sayed et al. (2001) Oncogene 20:6994]. We further showed that nocodazole-induced, p53 mediated activation of the JNK stress-activated kinases acts to inhibit nocodazole-triggered apoptosis. [Zhang et al. (2002) J. Biol. Chem. 277:43648]. In the present study, we sought to further investigate phosphorylation pathways that mediate the actions of inhibitors of microtubule dynamics such as nocodazole to arrest mitosis and induce apoptosis.

Materials & Methods

Total cell lysates were prepared from HeLa cervical and HCT-116 colon carcinoma cells following treatments with 200 ng/ml nocodazole for up to 72 h. Cells were washed with ice-cold PBS, scrapped in lysis buffer [20 mM Tris, 20 mM β-glycerophosphate, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml leupeptin, 4 μg/ml aprotinin and 1 μg/ml pepstatin A] and sonicated for 15 sec. Cell debris was removed by centrifugation at 100,000 rpm for 30 min at 4°C. Protein concentration was determined by the Bradford assay. For Kinetworks™ analysis, 350 μg of total protein were resolved on a 13% single-lane SDS-polyacrylamide gel, transferred to nitrocellulose membrane. Using a 20-lane multiblotter, the membrane was incubated with different mixtures of up to 3 antibodies per lane that react with a distinct subset of 25-50 different pan-specific or phospho-site-specific signalling proteins of distinct molecular masses. After further incubation with a mixture of relevant HRP conjugated secondary antibodies, the blots were developed using ECL and signals were quantified using Quantity One software (Bio-Rad, Hercules, CA). Detailed information and protocols of the Kinetworks™ analysis have been published [Pelech et al. (2003) Methods in Molecular Medicine Series: Cancer Cell Signalling Kinetworks™ protein kinase multiblot analysis. (D. Terrian, ed.) vol. 218, 99-111 Humana Press] and can also be found at the Kinexus Bioinformatics Corp. website (www.kinexus.ca). All other standard protocols presented in this study have been published [Zhang et al. (2002) J. Biol. Chem. 277:43648].

Results

In preliminary studies, we investigated the status of over 120 phosphorylation sites in human colon HCT116 cells treated for 20 h with 100 ng/ml of nocodazole using 5 different Kinetworks™ KPSS Phospho-site antibody screens. With nocodazole treatment, several proteins displayed 50% or more increases in phosphorylation including: Bad S136; CDK1 T161; FAK S722 and S910; Histone H3; NR1 S896; PKBα S473; PKCα T638 and S657; PKCε S719; PKCζ T410; PP1α T320; Raf1 S259; Rb S780; Src Y416; STAT1 S727; and STAT3 S727. Proteins that showed greater than 50% decreases in phosphorylation included: CDK1 T14/Y 15; eIF2α S52; FAK Y576, Y577 and Y925; Kit Y730; Lyn Y507; Mek1 T292 and S298; Met Y1003, Y1230 and Y1234; MLK3 T277; mTOR S2448, Pax Y31; PKCμ S916; Pyk2 Y579; and Rb T826. Figure 1 shows typical Kinetworks™ immunoblots with HeLa cell lysates, and several of these nocodazole induced changes (e.g. STAT3 S727 and Rb S780) were also evident in the human cervical cancer cells. There was also the altered phosphorylation of several phospho-site antibody cross-reactive proteins, including the nocodazole-induced increase of a 40 KDa protein (pp40) that was detected with a commercial antibody from Cell Signaling Technology that targets the phospho-Ser-217 and Ser-221 sites in the MAP kinase kinases Mek1 and Mek2. The remainder of our studies focused on the identification of pp40 and determination of its physiological role.

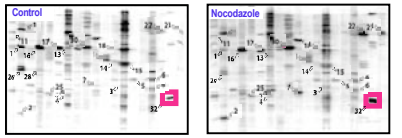

FIG. 1. HeLa cell phosphoproteins tracked in a Kinetworks KPSS 1.2 Phospho-site Screen.

1. Adducin - S724	9. MEK3/6 - S189/S207	17. PKCα - T638
2. CDK1 - Y15	10. MSK1 - S376	18. PKCδ - T505
3. CREB - S133	11. NR1 - S896	19. PKCε - S719
4. ERK1/2 - T202/Y204	12. p38α - T180/Y182	20. PKR - T451
5. Gsk3α/β - S21/S9	13. p70 S6K - T389	21. Raf1 - S259
6. Gsk3α/β - Y279/Y216	14. PKBα - T308	22. RB1 - S780
7. cJun - S73	15. PKBα - S473	23. RB1 - S809/S811
8. MEK1 - S217/S221	16. PKCα - S657	24. Rsk1 - T360/S364
		25 SAPK - T183/Y185
		26. Smad1 - S463/S465
		27. Src - Y418
		28. Src - Y529
		29. STAT1 - Y701
		30. STAT3 - S727
		31. STAT5 - S694
		32. pp40

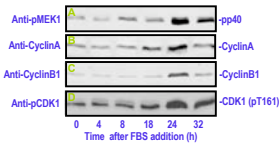

FIG. 2. pp40 is a mitosis-associated protein. Twenty-four hours after serum starvation, fetal bovine serum was added back to medium to induce HeLa cells to enter the cell cycle synchronously. The pp40 signal in cell lysates was detected by immunoblotting with anti-phospho-MEK1/2 antibody (A), and cell cycle progression was monitored by immunoblotting with antibodies for cyclin A (B), cyclin B1 (C) and phospho-CDK1 (pThr161) (D). Similarly, pp40 was also detected in HEK 293, A549 and MCF-7 cells by the same antibody with higher abundance in mitotic cells than interphase cells (data not shown), indicating that the increase of pp40 was generally associated with mitosis.

MEK1/2: ^{213}QLIDSMANSFVGTRSYMSPERLQ^{235}

B23WT: ^{1}MEDSMDMDMSPLRPQNYLFGCE^{22}

FIG. 3. pp40 is B23. Following protein purification and MALDI-TOF mass spectrometry of tryptic fragments, pp40 was identified as the abundant nuclear phosphoprotein B23, also known as nucleophosmin. From alignment of the phospho-epitope for the MEK1/2 antibody with B23, Ser-4 was putatively identified as the phosphorylation site.

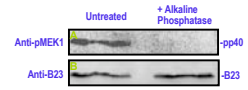

FIG. 4. Anti-pMEK1 recognizes phosphorylated B23 only. Incubation of total lysates of nocodazole-treated HeLa, A549 and MCF7 cells with 35 U alkaline phosphatase resulted in the disappearance of pp40 (A) whereas no effect was seen on B23 level (B).

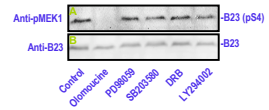

FIG. 5. B23 Ser-4 phosphorylation in HeLa cells in response to nocodazole is blocked by the cyclin-dependent kinase 1 (CDK1) inhibitor olomoucine, but not by inhibitors of MEK1, p38 MAP kinase, casein kinase 2 or PI 3-kinase. While no effect was seen when cells treated with PD 98059 (30 μM), SB 203580 (5 μM), DRB (20 μM) or LY 294002 (10 μM) along with nocodazole (100 ng/ml) for 20 h, treating with olomoucine (100 μM) completely abolished the B23 Ser-4 phosphorylation (A). Lysate from cells treated with nocodazole only was used as the control. No change of B23 protein level was found upon inhibitor treatment (B).

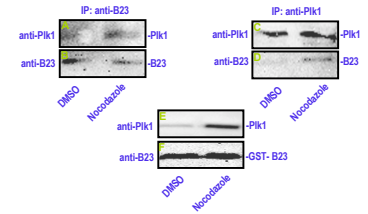

FIG. 6. B23 and Plk1 physically interact. B23 or Plk1 was immunoprecipitated from lysates from DMSO or nocodazole-treated HeLa cells using their respective antibodies, and the levels of target proteins in immunoprecipitates were examined by immunoblotting with the same antibodies (B, C). The presence of Plk1 or B23 was found in the immunoprecipitates of B23 or Plk1, respectively (A, D). A higher level of Plk1 was detected in the GST tagged B23-WT pull-down from nocodazole-treated HeLa lysate than from DMSO control (E). The amount of GST-B23 protein used in the pull-down assays is shown (F).

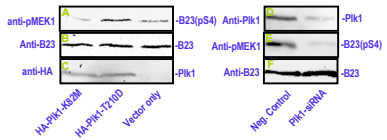

FIG. 7. Plk1 is B23 Ser-4 kinase in vivo. Transfection of HeLa cells with HA-Plk1-K82M (kinase-defective) resulted in a reduction in B23 Ser-4 phosphorylation while over-expression of HA-Plk1-T210D (active) enhanced the phosphorylation (A). No effect of overexpression on B23 protein level was found (B), and the expression of HA-tagged Plk1 proteins was detected by anti-HA antibody (C). Empty-vector transfected lysate was used as a control. Transfecting HEK293 cells with Plk1-siRNA resulted in the depletion of endogenous Plk1 protein (D), leading to a reduction of B23 Ser-4 phosphorylation (E). The B23 protein level was not reduced (F).

FIG. 8. Plk1 is a B23 Ser-4 kinase in vitro. Plk1 immunoprecipitate from lysates from nocodazole-treated HeLa cells was capable of phosphorylating in vitro GST-B23-WT with [γ-^{32}P]ATP, but not B23-S4A, as detected by autoradiography (A), and the phosphorylation of Ser-4 of GST-B23 by Plk1 immunoprecipitate could be detected by the phospho-MEK1/2 antibody (B). The level of Plk1 protein in Plk1 immunoprecipitates and the amount of GST-B23 proteins in the assays are shown in C and D, respectively.

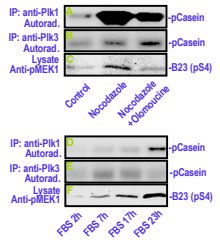

FIG. 9. Phosphorylation of B23 Ser-4 is specific for Plk1. The activities of Plk1 and Plk3 in HeLa cells upon the treatment of nocodazole with or without olomoucine were examined by subjecting Plk1 (A) or Plk3 (B) immunoprecipitates to in vitro [γ-^{32}P]ATP kinase assays using α-casein as a substrate followed by SDS-PAGE and autoradiography. Likewise, the activities of Plk1 and Plk3 during the cell cycle was monitored in HeLa cells sampled at various time points as indicated following the release from serum starvation (D,E). The phosphorylation of B23 Ser-4 was monitored in lysates using phospho-MEK1/2 antibody (C,F).

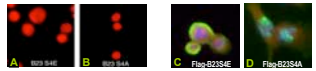

FIG. 10. Alterations of B23 Ser-4 phosphorylation results in aberrations in mitotic events in cells. Expression of B23S4E (D), but not B23S4A (not shown) nor pcDNA empty vector (A), in HEK 293 cells for 72 h after transfection induced the expansion of nuclear areas by ~80% (DAPI stained in red). HeLa cells transfected with Flag-tagged B23-S4E (C) or B23-S4A (D) were fixed at 48 h post transfection and stained with anti-Flag (green), anti-γ-tubulin (red) and DAPI (blue). B23-S4E-expressing cells contained up to 6 centrosomes (C), whereas B23-S4A over-expressed cells had one or no centrosomes and featured elongated, fragmented nuclei (D).

Conclusions

Plk1 phosphorylates Ser-4 of B23/nucleophosmin during the cell cycle and this facilitates centrosome duplication. Disruption of this event could lead to inhibition of tumour cell proliferation and validates Plk1 as a potential drug target for chemotherapy.

Acknowledgements - We are most grateful to Dr. Kenji Fukasawa (University of Cincinnati), Dr. Raymond Erikson (Harvard University, Cambridge, MA), and Dr. Wei Dai (New York Medical College, Valhalla, NY) for B23, Plk1, and Plk3 constructs, respectively.