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Identification of kinases phosphorylating 13 sites in the nuclear, DNA-binding protein NUCKS

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ABSTRACT

NUCKS is a vertebrate specific, nuclear and DNA-binding phospho protein. The protein is highly expressed in rapidly dividing cells, and is overexpressed in a number of cancer tissues. The phosphorylation of NUCKS is cell cycle and DNA-damage regulated, but little is known about the responsible kinases. By utilizing *in vitro* and *in vivo* phosphorylation assays using isolated NUCKS as well as synthetic NUCKS-derived peptides in combination with mass spectrometry, phosphopeptide mapping, phosphphoamino acid analyses, phosphospecific antibodies and the use of specific kinase inhibitors, we found that NUCKS is phosphorylated on 11 sites by CK2. At least 7 of the CK2 sites are phosphorylated *in vivo*. We also found that NUCKS is phosphorylated on two sites by ATM kinase and DNA-PK *in vitro*, and is phosphorylated *in vivo* by ATM kinase in γ–irradiated cells.
All together, we identified three kinases phosphorylating 13 out of 39 *in vivo* phosphorylated sites in mammalian NUCKS. The identification of CK2 and PIKK kinases as kinases phosphorylating NUCKS *in vivo* provide further evidence for the involvement of NUCKS in cell cycle control and DNA repair.

1. Introduction

NUCKS is a vertebrate specific, nuclear protein expressed in a number of tissues and cell types [1–3]. The copynumber of the NUCKS protein varies from 1.2E + 04 to 1.5E + 05 in mouse tissues. The expression is higher in cancer tissues and proliferating cells and varies from 3.2E + 05 to 1.4E + 0.6 in human cells lines (J.Wisniewski, personal communication). NUCKS is soluble in 5% perchloric acid as is the case for histone H1 and the HMG (high mobility group) proteins. The amino acid composition resembles that of the HMGB family of proteins, but the primary structure shows no homology with these proteins [3]. Instead, NUCKS exhibit homology with RAD51AP1 over a stretch of 25 amino acids in the N-terminal end, and is now regarded as a RAD51AP1 paralogue [4]. Circular dichroic analysis of purified NUCKS from rat liver showed no or very low contents of conventional α-helices or β-sheets but rather the presence of type I β-turns [3]. Furthermore, the amino acid composition of NUCKS is typical for highly flexible proteins without a defined structure in solution, but with the capability to induce a structure upon binding to a partner [5].

Like the HMG proteins, NUCKS binds to random-sequence DNA *in vitro* [6,7], and exhibit preferential binding to T-loop DNA [4]. In proliferating cells, the NUCKS protein is exclusively located in the nuclei in interphase of the cell cycle, but is dispersed in the cytoplasm at mitosis [3,8]. We have previously shown that the nuclear import of NUCKS is an active transport mediated by a single NLS and importin α3 and α5 [8]. A NUCKS splice variant lacking the NLS has been isolated from human brain (AAV83925.1). A similar splice variant has also been reported in chimpanzee (XP_001162120.1.) and *Macaca mulatta* (ENSMMUP00000038171). Ectopic expression of the human splice variant in HEK 293 cells showed a cytoplasmic localisation, indicating that NUCKS also might have a function outside the nucleus [8].

The *NUCKS* gene, which is located on chromosome 1 (1q32.1) in man, is expressed more or less in most cell types and tissues including embryonic stem cells [3,9,10]. It has now been shown that NUCKS is a positive transcriptional regulator of insulin signalling, and depletion of NUCKS in mice has been reported to lead to obesity and impaired glucose homeostasis [11–13]. The protein works by regulating chromatin accessibility and RNA polymerase II recruitment to the promoters of the insulin receptor and other insulin pathway modulators [11,12]. It has also been shown that NUCKS is a novel Tat co-activator increasing Tat-mediated viral transcription on the HIV-1 LTR promoter [14]. NUCKS is upregulated in neurons induced to die by apoptosis [15], and it has been reported that the gene is over expressed in a number of cancer cells [16–22]. Hence, NUCKS can be regarded as a potential biomarker in several cancer types [13,22,23]. Recently it was shown

Abbreviations: NUCKS, nuclear, ubiquitous, casein kinase and cyclin kinase substrate; NLS, nuclear localization signal; CK2, casein kinase 2; DNA-PK, DNA activated kinase; PIKK, phosphatidylinositol 3-kinase-related kinase; ATM kinase, ataxia telangiectasia mutated kinase; ATR kinase, ATM and Rad3-related.

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that *Nucks1* synergizes with Trp53 to promote radiation lymphomagenesis in mice [24]. The mechanism for the genetic interaction with Trp3 is not known. In addition, it has been shown that NUCKS is important for DNA repair by homologous recombination and hence important for chromosome stability. NUCKS is also, as is the case for a number of proteins involved in the HR pathway, important for replication fork progression [4].

Like p53, NUCKS is phosphorylated on multiple sites in proliferating cells *in vivo* [1,3,25]. In addition, it has been reported that NUCKS is acetylated, methylated, formylated and ubiquitinylated on multiple sites, implying that NUCKS has the highest ratio of modified to unmodified residues of any protein so far described [25] (Phosphosite Plus org.). Although the NUCKS protein seems to be multi-phosphorylated in all phases of the cell cycle, the phosphorylation of NUCKS from metaphase arrested cells is distinct from that observed in interphase cells, resulting in decreased mobility in both SDS and acetic acid/urea gels [1,3]. The decreased mobility can also be due to mitosis specific acetylation on Lys 175 and Lys 184 [25]. Unlike p53, the functional effect of phosphorylation of the different sites in NUCKS is not fully understood, and the kinases phosphorylating specific sites are not yet identified.

Protein phosphorylation constitutes an integral part of cell signalling and has a role in almost all cellular and developmental processes [26,27]. The knowledge of the protein kinases responsible for the phosphorylation of specific sites of a given protein is therefore of substantial importance. Amino acid motifs surrounding protein kinase phosphorylation sites are broadly classified as basic, acidic, proline-directed or phosphorylation of a tyrosine residue [28]. Delineation of minimal consensus sequences has improved the ability to detect new potential targets for specific protein kinases. However, there are limitations in the use of consensus sequences for the identification of such targets. There are sites that are efficiently phosphorylated by a specific kinase despite the fact that they do not match the phosphorylation motif for that kinase. Conversely, the presence of a minimal consensus sequence for phosphorylation by a specific kinase does not guarantee efficient phosphorylation, since there may be additional determinants within the sequence that modulate phosphorylation efficiency.

Various technologies have emerged to identify phosphoproteins and protein kinase substrates. Mass spectrometry is a powerful technology for proteomics and a method of choice for analysis of *in vivo* phosphorylation in different tissues and cells and also provides a valuable alternative to determine *in vitro* phosphorylation sites of purified proteins and peptides [29–35]. Although comparing *in vitro* phosphorylated samples and non-treated samples with phosphopeptide enrichment and mass spectrometry is a way to find substrates and phosphorylation motifs, these techniques alone do not always resolve the questions of which kinases that are responsible for the phosphorylation of specific substrates or sites.

Since the activation of most kinases is dependent on activation of specific signal transduction pathways, it is of significant interest to map the kinases responsible for phosphorylation of intracellular phosphoproteins with unknown or obscure functions. In this paper we have therefore by *in vitro* phosphorylation assays, utilizing both purified NUCKS from rat liver and synthetic NUCKS-derived peptides and purified kinases as well as cell extracts and specific kinase inhibitors, investigated putative kinases capable of phosphorylating several of the reported *in vivo* phosphorylated sites in NUCKS. We also investigated *in vitro* and *in vivo* CK2 phosphorylation sites by MALDI-MS and LC-MS. In addition, a phospho-specific antibody was generated and used to characterize a DNA-damage induced phosphorylation site in NUCKS.

2. Materials and methods

2.1. Materials

Purified DNA-activated kinase and recombinant CK2 were purchased from Promega. The p53 derived test peptide ($p53_{11-25}$) for DNA-activated kinase and ATM kinase (Biotin-EPPLSQEAFADLWKK) was purchased from Promega.

The synthetic CK2 test peptide (Biotin-C_4-DDDSDDD) was synthesized at The Biotechnology Centre, University of Oslo, Norway.

Recombinant GSK3 and CK1 were a kind gift from Laurent Meijer, Station Biologique. Cell Cycle Group, BP 74, 29682 Roscoff, France.

The CK2 inhibitor quinalizarin (Cat. no. 218717), the ATM kinase inhibitor KU 55933 (Cat.no 118502), the DNA-PK inhibitor NU 7441 (Cat. no. 3712) and the Chk1/2 inhibitor AZD hydrochloride (Cat. no. 5199) were from Tocris biosciences. All kinase inhibitors were solubilised in DMSO.

Protease inhibitor cocktail tablets were purchased from Roche.

The anti-ATM antibody was from Calbiochem (Ab-3, cat. No PC116). Anti-phospho p53 (S-15) and anti-phospho ATM (S-1981) antibodies were from Cell Signalling (#9284, #4526). The anti-p53 antibody (DO-1, sc-126) and Protein A/G PLUS-Agarose (sc-2003) were from Santa Cruz Biotechnology. Amersham horseradish peroxidase linked anti-mouse (NA-931) and anti-rabbit IgG (NA-934) were from GE Healthcare.

The M059K and M059J cells were a kind gift from Marie Dutreix, Institute Curie, France. The ATM proficient (C3ABR) and deficient (GM03189) B cells were a kind gift from Daniela Barila, Department of Biology, University of Rome.

The NUCKS derived peptides were synthesized at The Biotechnology Centre, University of Oslo, Norway and Eurogentec, and a phosphospecific anti-pSer54 NUCKS antibody was made by GenScript.

Synthetic NUCKS derived peptides:
Peptide A:
Biotin-C_{6-11}VDYSQFQESDDADED$_{25}$.
A_{mut} ($S_{19} \rightarrow A$):
Biotin-C_{6-11}VDYSQFQEADDADED$_{25}$.
Peptide B:
Biotin-C_{6-36}KIRSSPRE$_{43}$.
Peptide C:
Biotin-C_{6-51}GKNSQEDSEDSED$_{63}$.
C_{mut} ($S_{58} \rightarrow A$, $S_{61} \rightarrow A$):
Biotin-C_{6-51}GKNSQEDAEDAED$_{63}$.
Peptide D:
Biotin-C_{6-71}DDSHSAEDSEDE$_{82}$.
D_{mut} ($S_{75} \rightarrow A$, $S_{79} \rightarrow A$):
Biotin-C_{6-71}DDSHAAEDAEDE$_{82}$.
Peptide E:
Biotin-C_{6-109}EDVGSEEEQEEE$_{120}$.
Peptide F:
Biotin-C_{6-127}EKDSGSDEFL$_{136}$.
F_{mut1} ($S_{130} \rightarrow A$):
Biotin-C_{6-127}EKD**A**GSDEFL$_{136}$.
F_{mut2} ($S_{132} \rightarrow A$):
Biotin-C_{6-127}EKDSG**A**DEFL$_{136}$.
Peptide G:
Biotin-C_{6-139}EDDDDSDYG$_{147}$.
Peptide H:
Biotin-C_{6-177}TVTPSPVKGKGKVGRPTA$_{192}$.
Peptide I:
Biotin-C_{6-199}KEKTPSPKEEDEE$_{211}$.
Peptide J:
Biotin-C_{6-212}PESPPEKKT$_{220}$.
Peptide K:
Biotin-C_{6-221}STSPPPEKSGDE$_{232}$.
Peptide L:
Biotin-C6-$_{230}$GDEGSEDEAPSGED$_{243}$.
L_{mut1} ($S_{234} \rightarrow A$):
Biotin-C_{6-230}GDEG**A**EDEAPSGED$_{243}$.
L_{mut2} ($S_{240} \rightarrow A$):
Biotin-C_{6-230}GDEGSEDEAP**A**GED$_{243}$.