The major phosphorylation site of nucleophosmin (B23) is phosphorylated by a nuclear kinase II

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Nucleophosmin (B23) was phosphorylated *in vitro* with [γ - 32 P]ATP and a nuclear kinase (type II) purified from HeLa cells. The phosphorylation was inhibited by heparin and by 2,3-diphosphoglycerate. Peptide mapping analysis indicated that the phosphorylation site *in vitro* was identical to that *in vivo*. Purified nucleoli have a similar kinase that phosphorylated nucleophosmin at the same site. These results indicated that nucleophosmin is phosphorylated *in vivo* by a nucleolar kinase (type II).

INTRODUCTION

Phosphoproteins are involved in many cellular processes [1–3]. Increases in the phosphorylation of nuclear proteins have been correlated with chromatin template activity [4]. Several nucleolar phosphoproteins had been identified in proliferating and tumour cells [5]. Nucleophosmin (also termed B23, N038 and numatrin) is a major nucleolar phosphoprotein which is 20 times more abundant in tumour and growing tissues than in normal resting tissues [6–8]. Nucleophosmin is associated with pre-ribosomal particles [9,10] and forms hexamers [11] which may be essential for the assembly of ribosomes. The amino acid sequence of human nucleophosmin has been determined [7]. Highly conserved sequences were observed among nucleophosmins from *Xenopus laevis*, chicken, mouse, rat and human [12–15]. The N-terminal half [7] has 50% sequence identity with nucleoplasmin, a nucleosome assembly factor [16,17]. It was recently reported that nucleophosmin shuttles between the nucleus and cytoplasm [15] and that its threonine residues are highly phosphorylated by cdc2 kinase during mitosis [18].

In our previous studies, a major phosphorylation site (constituting about 80% of the total phosphorylated peptide) of nucleophosmin was identified which is located in the highly acidic region of the molecule [19]. The amino acid sequence of this phosphorylation site (P-Ser)-Glu-Asp-Glu-Asp-Glu-Glu-Asp-, is similar to those of nucleolar protein C23, the R-II subunit of cyclic AMP-dependent protein kinase and glycogen synthase [19]. These remarkably similar amino acid sequences in these different proteins strongly indicate that this site may have a common function. Phosphorylation sites within an acidic amino acid cluster are typically phosphorylated by casein kinase II [2,3]. To determine which kinase phosphorylates nucleophosmin *in vivo*, the current study compares the phosphorylation sites of nucleophosmin *in vivo* and *in vitro*. The results indicate that a nucleolar kinase (N-II) phosphorylates the major phosphorylation site of nucleophosmin *in vivo*.

MATERIALS AND METHODS

Isolation of nucleophosmin

HeLa S-3 cells were grown in Eagle's minimum essential medium supplemented with 10% newborn calf serum in a

humidified incubator containing 5% CO₂ in air at 37 °C. For 32 P-labelling, HeLa cells were incubated in medium containing 2 mCi of [32 P]P_i/100 ml for 16 h before harvest.

Nuclei were isolated from HeLa cells using the Nonidet P-40 method [11,20]. Nucleoli isolated from 4×10^6 cells were extracted with 1 ml of 3 M-urea/0.2 mM-phenylmethanesulphonyl fluoride/0.1 mM-*p*-chloromercuriphenylsulphonic acid/0.1 mM-leupeptin. The nucleoli suspension was incubated at 60 °C for 30 min and cooled in ice before centrifugation at 10000 *g* for 10 min. The supernatant contained purified nucleophosmin with about 80% purity, as determined by two-dimensional gel electrophoresis (Fig. 1a).

Phosphorylation of nucleophosmin *in vitro* by nuclear kinase II

Nuclear kinase (N-II) was purified from HeLa cell nuclei to near homogeneity using DEAE-Sephacel, phosphitin-Sephacel and phosphocellulose chromatography [21–23]. The purified kinase has properties typical of casein kinase type II [2,22]. The purified N-II kinase is devoid of nuclear kinase I activity [2] and has a specific activity of about 1 μ mol/min per mg using phosphitin as the substrate [22]. Purified nucleophosmin and N-II kinase were dialysed against 50 mM-Tris/HCl (pH 8.0)/50 mM-NaCl/10 mM-MgCl₂ (kinase buffer) before use. The kinase reaction mixture, which contained nucleophosmin, kinase and [γ - 32 P]ATP in kinase buffer in the presence or absence of inhibitors, was incubated at 37 °C for 5–30 min. Phosphorylated nucleophosmin was dialysed in 8 M-urea overnight before being subjected to two-dimensional gel electrophoresis. The two-dimensional gel was stained with Coomassie Blue, dried and exposed to X-ray film. The Coomassie Blue-stained nucleophosmin was excised from gels and the incorporation of 32 P into the nucleophosmin was measured by scintillation counting.

Isolation and characterization of phosphopeptides

The methods for the isolation and characterization of phosphopeptides have been described previously [19]. Nucleophosmin was purified by two-dimensional gel electrophoresis (Fig. 1a). The Coomassie Blue-stained nucleophosmin spot was excised from the gels and digested with trypsin. The phosphopeptides were separated on a thin layer of cellulose by electrophoresis at pH 2.3 (first dimension) and ascending chromatography (second dimension).

Abbreviations used: 2,3-DPG, 2,3-diphosphoglycerate; N-II kinase, nuclear kinase II.

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Fig. 1. Nucleophosmin phosphorylation *in vitro*

Nucleophosmin and N-II kinase were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in kinase buffer. Nucleophosmin was then separated by two-dimensional gel electrophoresis. (a) Coomassie Blue stain (IF, isoelectric focusing); (b) autoradiogram of the gel; (c) incorporation of ^{32}P into nucleophosmin as determined by scintillation counting. Incubation was carried out for 5–30 min. +Hep, in the presence of heparin (20 $\mu\text{g}/\text{ml}$); +DPG, in the presence of 2,3-DPG (15 mM).

Fig. 2. Tryptic phosphopeptide map of nucleophosmin

HeLa nucleophosmin was excised from two-dimensional gels and digested with trypsin. The tryptic phosphopeptides were analysed by two-dimensional peptide mapping (+, origin; E, electrophoresis direction from positive to negative poles; C, ascending chromatography direction). (a) Nucleophosmin phosphorylated *in vivo*; (b) nucleophosmin phosphorylated *in vitro* by N-II kinase; (c) mixture of (a) and (b); (d) nucleophosmin phosphorylated *in vitro* from the purified nucleoli of HeLa cells; (e) nucleophosmin phosphorylated *in vitro* by cyclic AMP-dependent protein kinase.

RESULTS AND DISCUSSION

Phosphorylation of nucleophosmin *in vitro* by N-II kinase

Nucleophosmin purified from HeLa cells was phosphorylated *in vitro* by HeLa N-II kinase. Fig. 1(a) shows the Coomassie Blue-

stained nucleophosmin (arrow), which is the major phosphorylated protein as determined by autoradiography (Fig. 1b). Fig. 1(c) shows that the phosphorylation of nucleophosmin increased with incubation time (from 5 to 30 min). A total of 97% and 89% of the phosphorylation could be inhibited

by addition of heparin (20 $\mu\text{g/ml}$) or 2,3-diphosphoglycerate (2,3-DPG, 15 mM) respectively after 30 min of incubation. Inhibition by these two agents was also observed when phosphorylation was conducted in nucleoli (which contain both endogenous nucleophosmin and kinase). Because N-II kinase is sensitive to heparin and to 2,3-DPG [2], this study indicates that the N-II kinase could be the major endogenous nuclear kinase that phosphorylates nucleophosmin.

Identification of phosphorylation sites of nucleophosmin

To investigate whether N-II kinase phosphorylates nucleophosmin *in vitro* at the same site as that phosphorylated *in vivo*, the phosphopeptide maps were compared. Nucleophosmin labelled *in vivo* was isolated from HeLa cells, and nucleophosmin labelled *in vitro* was obtained from the N-II kinase reaction. Labelled nucleophosmins were purified from two-dimensional gel electrophoresis, digested with trypsin and analysed by two-dimensional peptide mapping. Fig. 2(a) shows the phosphopeptide map of nucleophosmin labelled *in vivo*. A major phosphopeptide was observed (arrowhead) which constituted about 80 % of the total radioactivity. This phosphopeptide labelled *in vivo* is identical to that labelled *in vitro* with N-II kinase (Fig. 2b). The identity of this peptide was demonstrated by the co-migration in the mixed sample of peptides produced *in vivo* and *in vitro* (Fig. 2c). This major peptide is the same as the acidic phosphopeptide (P-peptide) previously identified in Novikoff hepatoma [19].

We obtained the same phosphopeptide *in vitro* (Fig. 2d) when phosphorylation was carried out in purified nucleoli without the addition of exogenous kinase. The phosphorylation in nucleoli was also inhibited by heparin (20 $\mu\text{g/ml}$). Results of these studies indicate that nucleoli contain the endogenous N-II kinase.

When nucleophosmin was phosphorylated *in vitro* by cyclic AMP-dependent protein kinase (Sigma, cat. no. P2645), a phosphopeptide map completely different from that obtained with the N-II kinase was produced (Fig. 2e). These results suggest that the major phosphorylation site was not phosphorylated by cyclic AMP-dependent protein kinase. We have shown that serine is the phosphorylated amino acid which is inside the acidic amino acid cluster of nucleophosmin [7,19]. A different phosphorylation site was recently reported by Peter *et al.* [18], in which the threonine residue was highly phosphorylated during mitosis by cdc2 kinase. In the tryptic map *in vivo* (Fig. 2a) there are minor phosphopeptides [19], one of which may be the mitotic phosphopeptide.

To investigate the possibility of autophosphorylation, nucleophosmin was purified by an antibody affinity column [11]. The protein was eluted from the affinity column with 4 M-MgCl₂, pH 5.4, which is a relatively mild reagent, in order to preserve the enzyme activity (if any). Affinity-purified nucleophosmin was incubated with [γ -³²P]ATP without exogenous kinase. No incorporation of ³²P into nucleophosmin was observed, which rules out the occurrence of autophosphorylation (results not shown).

Possible function of the phosphorylation of nucleophosmin

The current studies indicate that the major phosphorylation site of nucleophosmin is phosphorylated by a nucleolar kinase (type II). Our results are in good agreement with those of Pfaff & Anderer [24], who reported that nucleoli contain high concentrations of N-II kinase. Phosphorylation by N-II kinase and subsequent stimulation of the enzyme activities of topoisomerase I [21], topoisomerase II [25] and RNA polymerase I [26] were reported. As pointed out by Hathaway & Traugh [2] N-II kinase is a multisubstrate kinase, and may have multiple functions. Phosphorylation of these proteins by N-II kinase may have a co-operative role in cellular activity. Preliminary experiments

indicated that the phosphorylation site of nucleophosmin remains unchanged during serum starvation and actinomycin D treatments (results not shown). We tentatively conclude that phosphorylation of nucleophosmin by N-II kinase is not part of the phosphorylation/dephosphorylation regulation process.

The function of the phosphate in nucleophosmin is not known. The amount of nucleophosmin increases during cell proliferation. It is believed that the hexameric nucleophosmin [11] serves to deliver ribosomal proteins and provide the structural support necessary for the interaction of individual ribosomal proteins and the newly synthesized rRNA. Recent studies using mutagenesis suggested that the acidic cluster region which included the phosphorylation site was in the periphery of the molecule and did not affect the oligomerization of nucleophosmin (Q.-R. Liu & P. K. Chan, unpublished work). It is conceivable that temporary binding of the acidic cluster to the basic ribosomal proteins will stabilize the charge of the preribosomal particle structure so that ribosomal proteins can be incorporated into ribosomes. The phosphate group of nucleophosmin may enhance the binding of the protein.

This work has supported by USPHS grant CA 42476 awarded by the National Cancer Institute (to P.K.C) and in part by a grant from the Robert A. Welch Foundation (to E.D.). We thank M. Aldrich for her excellent technical help.

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Received 10 May 1990/28 June 1990; accepted 3 July 1990