Inhibition of nucleoside diphosphate kinase activity by in vitro phosphorylation by protein kinase CK2

Differential phosphorylation of NDP kinases in HeLa cells in culture

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Abstract Although a number of nucleoside diphosphate kinases (NDPKs) have been reported to act as inhibitors of metastasis or as a transcription factor in mammals, it is not known whether these functions are linked to their enzymatic activity or how this protein is regulated. In this report, we show that in vitro protein kinase CK2 catalyzed phosphorylation of human NDPK A inhibits its enzymatic activity by inhibiting the first step of its ping-pong mechanism of catalysis: its autophosphorylation. Upon in vivo ^{32}P labeling of HeLa cells, we observed that both human NDPKs, A and B, were autophosphorylated on histidine residues, however, only the B isoform appeared to be serine phosphorylated.

Key words: NDP kinase; nm23; Protein kinase CK2; Enzyme inhibition; Histidine phosphorylation

1. Introduction

Nucleoside diphosphate kinase (NDPK) (EC 2.7.4.6) is a ubiquitous enzyme that catalyzes the transfer of a terminal phosphate from oxy- or deoxy-NTPs to oxy- or deoxy-NDPs following a ping-pong mechanism according to the reaction:

$$N_1TP + E \Leftrightarrow E{-}P + N_1DP$$

$$E - P + N_2DP \Leftrightarrow E + N_2TP$$

where N_1TP is physiologically ATP [1]. In the first step of the reaction, the enzyme is phosphorylated in an Nδ position of a strictly conserved histidine of the active site [2]; this intermediate of the reaction is stable in the absence of an acceptor N_2DP.

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Abbreviations: NDPK, nucleoside diphosphate kinase; CK2, protein kinase CK2 (formerly called casein kinase 2); DMEM, Dulbecco's modified Eagle's medium.

The NDPKs crystallized so far have been found to be hexamers [3–5], with the exception of the *Myxococcus xanthus* enzyme which has a tetrameric structure [6]. In lower eukaryotes, the enzyme is composed of six identical subunits whereas in vertebrates and plants at least two different subunits have been found [1,7]. Human NDPK is a hexamer composed variably of two different subunits (A and B) which share 88% identity, exhibit different p*I* values and electrophoretic mobilities [8] and have a different intracellular localization; while the two isoforms are found in the cytoplasm, only NDPK B has been described as having a nuclear localization [9].

The classical role ascribed to the NDPKs has been to maintain the intracellular NTPs pool [1]. More recently, human NDPK A and murine NDPKs α and β (encoded by nm23-H1, nm23-M1 and nm23-M2, respectively) have been characterized as inhibitors of metastasis [10–12]. Also, human NDPK B has been identified as 'PuF', a factor that stimulates transcription of the proto-oncogen c-myc [13,14]. However, at present, it is not clear how these processes are regulated.

An important autophosphorylation on serine residues was claimed to occur in several NDPKs [15–17], and it was suggested that serine phosphorylation could correlate with the inhibition of metastasis [16]. Nevertheless, the importance of serine autophosphorylation is in doubt since results obtained using methodologies that unambiguously recognize non-histidine phosphorylation, as shown by Bominaar et al. [18] and by us [19], clearly demonstrated that autophosphorylation on serine residues could only account for less than 0.2% of the total autophosphorylation.

We have previously shown that NDPKs from different origins are in vitro substrates of recombinant human protein kinase CK2 (rhCK2) [20], however, the potential action of this phosphorylation on enzymatic activity was not investigated. In the present work, we used recombinant human NDPK A (rhNDPK A) as the main model for studying the effect of phosphorylation by rhCK2 on enzyme activity. We present evidence that phosphorylation of the enzyme by CK2 inhibits its overall enzymatic activity by inhibiting the first step of the reaction, i.e. autophosphorylation. To reinforce the idea that phosphorylation by CK2 could represent a regulatory mechanism of NDPK activity we investigated whether NDPKs from HeLa cells could be phosphorylated in vivo on serine residues.

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2. Materials and methods

2.1. Materials

Recombinant human protein kinase CK2, and NDPK A were prepared as described [20,21]. RhNDPK B was obtained according to [22]. [γ-^{32}P]ATP was obtained from New England Nuclear; carrier-free [^{32}P]orthophosphate was obtained from the Comisión Nacional de Energía Atómica, Buenos Aires, Argentina. Protein A-Sepharose and okadaic acid were from Sigma. Polyacrylamide gel electrophoresis reagents were from Bio-Rad. X-ray films (Curix-RP1) were from Agfa Gevaert. Immobilon-P transfer membranes were from Millipore. All other reagents were of analytical grade.

2.2. In vitro phosphorylation of recombinant human NDPK A by CK2

RhNDPK A was phosphorylated essentially as described in [20] except that, in experiments aimed to measure enzymatic activity or autophosphorylation of NDPK, CK2 catalyzed phosphorylation was performed with unlabeled ATP. A control in which rhCK2 was not added was performed in parallel. At the times indicated, aliquots were taken from the incubated CK2 and control tubes, the reaction was stopped by the addition of 40 μg/ml heparin and the samples were kept on ice until processing.

2.3. Assay of NDPK activity

Enzymatic activity was assessed by incubating the samples for 10 min at 30°C as described [23] using pyruvate kinase as a coupled enzyme and measuring the pyruvic acid formed using the 2,4-dinitrophenylhydrazone method [24]. Briefly, the standard incubation mixture contained 50 mM Tris-HCl, pH 7.5, 2 mM ATP, 2 mM dTDP, 5 mM $MgCl_2$, 60 mM KCl, 0.2 units of pyruvate kinase and an appropriate dilution of the enzyme (made in BSA buffer containing: 50 mM Tris-HCl pH 7.5, 0.5 mg/ml BSA) in a final volume of 0.1 ml. 1 unit of NDPK activity was defined as the amount of enzyme that catalyzed the transfer of 1 μmol of phosphate from ATP to dTDP per min under these conditions.

2.4. Assay of NDPK autophosphorylation

Autophosphorylation assays were performed at 4°C in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 10 mM EDTA, 100 μM [γ-^{32}P]ATP (10 mCi/μmol) and an appropriate dilution of the enzyme (made in BSA buffer) in a final volume of 10 μl. After 10 min incubation, the reaction was stopped with 1 vol. of 2×Laemmli sample buffer [25], and heated for 2 min at 45°C. The products were separated by 15% SDS-PAGE according to Laemmli [25] and visualized by autoradiography of the dried gels after overnight exposure.

2.5. Metabolic labeling of cultured HeLa cells and immunoprecipitation of NDPKs

HeLa cells were maintained as described by Rosenblatt et al. [26]. For experimental purposes, cells were plated into 60 mm Petri dishes in Dulbecco's modified Eagle's medium supplemented with low molecular weight nutrients and 5% fetal calf serum. 2 days later, the cultures received fresh medium containing only 100 μM phosphate and 5% dialyzed fetal calf serum. Under such conditions, HeLa cells exhibit continuous division. For NDPK phosphorylation studies, cells were incubated with 300 μCi of carrier-free [^{32}P]orthophosphate 3 h prior to stimulation to allow steady-state labeling of the endogenous ATP pool. The culture was then stimulated for 2 h with 10% dialyzed fetal calf serum. Okadaic acid (1 μM) was added to the culture medium for the last 15 min. Cells were then rinsed twice in a solution containing 20 mM Tris-HCl, pH 7.5 and 0.15 M NaCl, at 4°C; the extracts were prepared with the addition of a chilled solution containing 10 mM Tris-HCl, pH 7.6, 5 mM NaF, 100 μM orthovanadate, 1 mM PMSF, and 1% Nonidet P40. Cell extracts were centrifuged at 10000 rpm for 30 min in a refrigerated microcentrifuge. The supernatant was used immediately for immunoprecipitation of NDPKs as described [19] except that the antibodies used were a mixture of 4 different monoclonal anti-NDPK antibodies to ensure immunoprecipitation of both NDPK isotypes. The antibodies used were mAb 37.6, 10-2, and 126-1, which recognize native and denatured A and B subunits with high affinity, and mAb 143-2, which recognizes the quaternary conformation of NDPKs from different origins. The incubation time was 1 h.

2.6. Evaluation of acid and base stability of in vivo phosphorylated NDPKs on Immobilon membranes

The methodology used was as described [19], employing 3 h incubations in the different pH buffers. Under these conditions, phosphate bound to histidine residues is depleted during incubation at pH 1, while that bound to serine/threonine remains. In contrast, incubation at pH 14 depletes phosphates bound to serine/threonine residues while histidine phosphorylation is stable under these conditions.

3. Results and discussion

3.1. Effect of rhCK2 phosphorylation on rhNDPK enzymatic activity and autophosphorylation

To investigate whether in vitro phosphorylation by CK2 produces any change in NDPK enzymatic activity, it was important to ensure a high degree of phosphorylation of NDPK. Previous studies indicated that, among the NDPKs which served as substrates for CK2, NDPK A was the most efficiently phosphorylated, being a 2.5-times better substrate than the B counterpart [20]. A time course experiment was performed as detailed under Section 2 using rhNDPK A as substrate. Aliquots obtained from CK2 incubated (+CK2) and from controls devoid of CK2 (−CK2) were diluted and tested for NDPK activity (Fig. 1A). As can be seen, time-

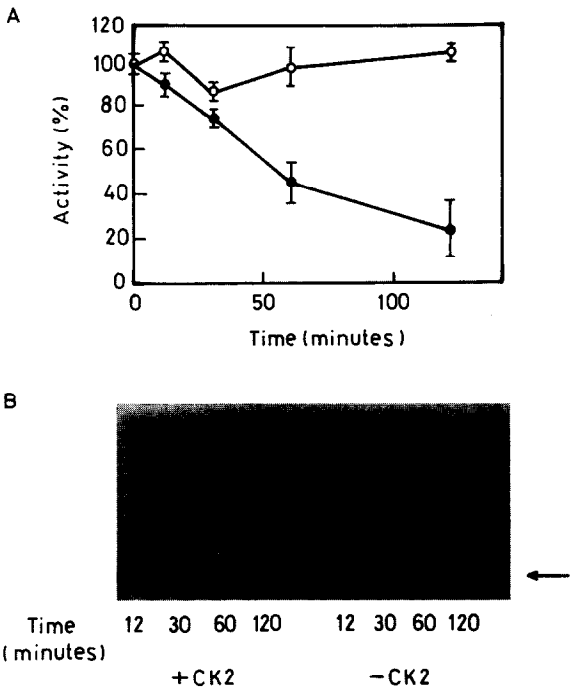

Fig. 1. Activity and autophosphorylation of rhNDPK A as a function of phosphorylation by rhCK2. RhNDPK A was phosphorylated in a time course experiment (●—●, +CK2) or incubated in the reaction mixture in the absence of CK2 as control (○—○, −CK2) as described under Section 2. (A) Remnant NDPK activity of rhCK2-phosphorylated rhNDPK A. Aliquots of rhNDPK A with different extents of phosphorylation (●—●) and controls not phosphorylated (○—○) were diluted (more than 2000-fold) and tested for NDPK activity. All activities are referred to zero time taken as 100% (300 units/mg at 30°C). Error bars indicate the standard deviation from three different dilutions from the original sample. (B) Autophosphorylation of CK2 phosphorylated rhNDPK A. Equal amounts of rhNDPK A from CK2 treated samples with different extents of phosphorylation (+CK2) or control experiments (−CK2) were taken at the times indicated and assessed for autophosphorylation as described in Section 2. The arrow indicates the position of rhNDPK A.

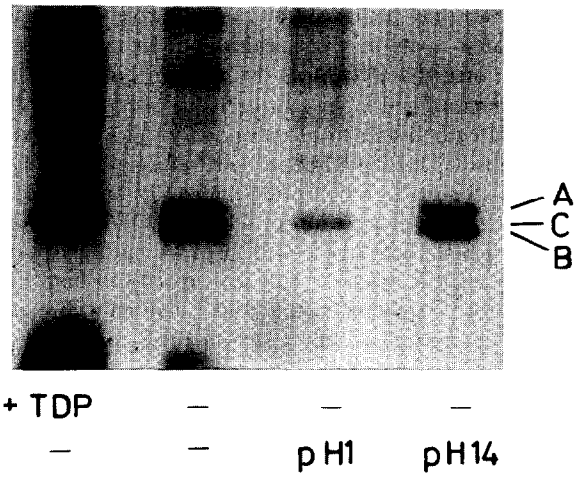

Fig. 2. In vivo phosphorylation of NDPKs. Acid and base stable phosphorylation of in vivo labeled NDPKs. NDPKs were immunoprecipitated from crude extracts of HeLa cells labeled in vivo with ^{32}P as described in Section 2. Protein A-Sepharose-bound NDPK immunocomplexes were resuspended in sample buffer directly or after incubation in buffer containing 10 mM Mg^{2+}·TDP (5 min at room temperature), separated by SDS-PAGE and transferred to Immobilon membranes. The sample incubated with Mg^{2+}·TDP (lane 1) had approx. 15 times more protein loaded than the other lanes (which have exactly the same amount). Immobilon stripes were either incubated at pH 14 (lane 4), pH 1 (lane 3) or not treated (lane 2) in order to determine the type of phosphorylated residue present in immunoprecipitated NDPK. A and B indicate the positions of autophosphorylated NDPKs A and B, respectively; C denotes the position of serine phosphorylated NDPK. The labeled bands of higher molecular mass probably represent dimers or trimers of NDPK, resulting from incomplete denaturation and/or disulfide cross-linking.

dependent inactivation of NDPK occurs in samples preincubated with CK2 compared with controls devoid of CK2. Maximal inhibition, around 80%, was achieved after 2 h incubation with CK2. In phosphorylation experiments performed in parallel using [γ-^{32}P]ATP, the maximum CK2-catalyzed phosphorylation of rhNDPK A attained was approx. 0.8 mol phosphate per mol of monomer after 2 h incubation (not shown). When rhNDPK B was used instead of rhNDPK A in CK2 phosphorylation experiments, time-dependent inhibition of enzyme activity was also observed, reaching 30% inhibition after 2 h preincubation with CK2; this inhibition also correlated with the degree of phosphorylation of the enzyme in parallel experiments. These data indicate a close relationship between the degree of phosphorylation of NDPKs and the inhibition of enzymatic activity.

To assess whether phosphorylation of NDPK by CK2 affects the first step of the NDPK-catalyzed reaction, autophosphorylation assays were performed with the same rhNDPK A aliquots that had been incubated with or without CK2 for different periods of time. As Fig. 1B shows, the control experiments demonstrated no differences in their autophosphorylation pattern, while there was a marked inhibition in the CK2-treated samples. Since compounds such as Mg^{2+}, NaCl and polylysine are necessary components to accomplish phosphorylation of NDPK by CK2, and since they affect the equilibrium of the first step of the NDPK reaction (R.M.B. and S.P., unpublished observations), care was taken to perform autophosphorylation assays with diluted samples (more than 100-fold) in order to avoid any interference from these compounds.

Taken together, these results show that the inhibition of NDPK enzymatic activity is due to the fact that the CK2-phosphorylated enzyme is probably unable to perform the first step of its ping-pong reaction. These results also imply that there is probably only one phosphorylation site per subunit. In our previous report, we showed that phosphoserine was the only phosphoamino acid found after acid hydrolysis of CK2-phosphorylated NDPK and two serine residues were mapped as the possible phosphorylation sites: Ser +4 and/or Ser +7 with respect to the histidine of the active site. Either residue, if phosphorylated, might affect the overall charge environment of the active histidine, presumably altering the necessary conditions for effective nucleophilic attack on the NTP terminal phosphate. These Ser residues are conserved in all NDPKs characterized so far except *Drosophila* NDPK in which Ser +4 is replaced by Ala. Interestingly, we have shown that *Drosophila* NDPK was not phosphorylated in vitro by CK2 [20].

3.2. *In vivo labeling of NDPKs from HeLa cells in culture*

To examine the possibility that serine phosphorylation could be a physiological means of regulation of NDPKs, HeLa cells were metabolically labeled with [^{32}P]orthophosphate and NDPKs were immunoprecipitated from whole extracts. The immunoprecipitates were resuspended in buffer with or without Mg^{2+}·TDP, the proteins being separated in 15% SDS-PAGE and blotted onto Immobilon membranes. Serine/threonine phosphorylation of NDPKs was assessed using an improved methodology which allows reliable discrimination between histidine and serine/threonine phosphorylation [19]. An autoradiograph of such an experiment is shown in Fig. 2. In lane 2, where the immunoprecipitated NDPK was not treated, two phosphorylated bands are clearly observed, corresponding to NDPK A and B (indicated as bands A and B). When a strip of the Immobilon membrane was treated in buffer of pH 14 (lane 4) (where His-P is stable

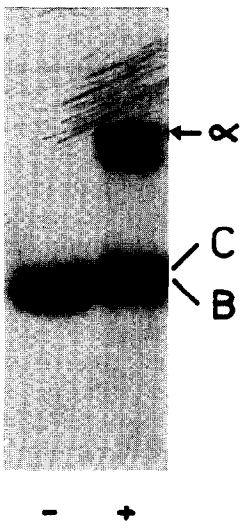

Fig. 3. In vitro phosphorylation of rhNDPK B. Phosphorylation by CK2 for 1 h was performed as described [20]. The control lane shows autophosphorylated NDPK B. Autophosphorylation was performed in the presence of 10 mM EDTA and 0.5 mM $MgCl_2$. α indicates the position of the corresponding subunit of CK2.

and Ser-P/Thr-P are not), the two bands corresponding to NDPK A and B were found to be still phosphorylated, revealing that they were histidine phosphorylated. On the other hand, when another strip was treated at pH 1 (where Ser-P/Thr-P are stable and His-P is not), a less intensely serine/threonine phosphorylated band was made visible at a position slightly above that of the histidine phosphorylated NDPK B (lane 3). This same band (labeled C) was again made visible when the immunoprecipitated NDPKs were incubated with Mg^{2+}·TDP (lane 1); in this lane, the histidine phosphorylated NDPK A and B disappeared, proving that they were the histidine phosphorylated high-energy intermediates of the reaction. This third band (C) most probably represents the serine phosphorylated form of NDPK B; the same slight retardation effect on SDS gel migration was also observed when purified rhNDPK B was phosphorylated by CK2 in vitro (Fig. 3). As expected, the proximity of the two bands leads to a single merged band spanning B and C when blotted with antibodies against NDPK B (not shown). The fact that phosphatase inhibitor cocktails are rarely included in extract preparations and the proximity of the two bands in 15% SDS-PAGE may be reasons explaining why a third human NDPK band had not been previously observed using this methodology. In addition, in the experiment presented here, the intensity of band C is very minor in comparison to that of bands A and B and thus may be clearly visible only via a sensitive methodology that eliminates the background of band B.

In conclusion, our demonstration of in vivo serine/threonine phosphorylation of NDPK together with the measured inhibitory effect of in vitro CK2 phosphorylation on NDPKs activity suggests that this phosphorylation could be a mechanism to regulate NDPK activity, e.g. in relation to the cell cycle. NDPK activity, which normally has a high turnover rate of up to 1000 s^{-1}, could be partially inhibited by serine phosphorylation in resting cells. Upon mitogenic stimulation, the need for large amounts of NTPs could be satisfied rapidly via phosphatase-catalyzed dephosphorylation of NDPK, thus raising NDPK activity.

It has been observed that NDPK expression correlates with proliferation in a variety of human cell models [27–29]; also, NDPK activity correlates with proliferation of *C. albicans* yeast cells [23]. In general, de novo expression of NDPK in stimulated cells has been shown more clearly at the mRNA than at the protein level, indicating that relatively high levels of NDPK protein were already present in the resting cells, almost masking the newly synthesized protein. In the fission yeast *Schizosaccharomyces pombe* a post-translational regulation of NDPK enzymatic activity has been suggested [30]. Before entering the S-phase, the NDPK activity was doubled in step-wise fashion, without evidence of the de novo synthesis of NDPK protein. In this context, it would be interesting to know whether in vivo phosphorylation of NDPK subunits is cell cycle related and more abundant serine phosphorylated species could be detected in a particular cycle phase. It should be noted that HeLa cells have been a useful model to determine the existence of serine phosphorylated NDPK, but because cells are not synchronized, the possibility remains that phosphorylation represents a minority of cells at a specific stage of the cell cycle.

Nevertheless, it is intriguing that only NDPK B, and only a fraction of the total, was found to be serine phosphorylated in vivo. This fact may point to a more subtle role of phosphorylation related to its in vivo localization. In this respect, it should be noted that NDPK B differs from NDPK A in its ascribed functions and in vivo intracellular distribution. While both have a cytoplasmic localization, only the B isoform has been described as having a nuclear localization [8], correlating with its function as a transcription factor [13] and co-localizing with the major population of CK2 [31]. Moreover, it remains unexplained why the NDPK B from partially purified from HeLa nuclear extracts, with the capacity to bind the c-myc promotor [13], eluted from a DEAE column with a greater negative charge than would have been predicted from its p*I*. Phosphorylation could indeed be responsible for this behaviour. Nevertheless, one cannot rule out the possibility that NDPK B is in vivo phosphorylated by another kinase able to distinguish between the two isoforms of NDPK.

Recently, NDPK B with a Ser→Gly mutation at position 122 (+4) has been isolated from a malignant melanoma cell line [32]. This mutation has no influence on the enzyme activity; however, based on the results presented here, the hypothesis may be raised that the Ser→Gly 122 mutation may affect NDPK regulation through phosphorylation. Hence, removal of an inhibitory phosphorylation site in NDPK might also be important for tumorigenesis in some cases.

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