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Regulation of Cellular Functions by Nucleoside Diphosphate Kinases in Mammals

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The role of nucleoside diphosphate (NDP) kinases in cell growth, differentiation, and tumor metastasis in relation to signal transduction was investigated. The essential role of NDP kinase in cell growth was validated by coupling between reduced NDP kinase levels, induced by antisense oligonucleotides, and the suppression of proliferative activity of a cultured cell line. In addition, because NDP kinase levels are often enhanced with development and differentiation, as has been demonstrated in postmitotic cells and tissues, such as the heart and brain, we further examined this possibility using the bone tissue (osteoblasts) and a cultured cell line PC12D. The enhanced NDP kinase accumulation was demonstrated in the matured osteoblasts *in vivo* and *in vitro* by immunohistochemistry. In PC12D cells, neurite outgrowth took place in NDP kinase β-transfected clones without differentiation inducers, which was accompanied by prolongation of doubling time. Neurite outgrowth, triggered by nerve growth factor and a cyclic AMP analog, was down-regulated upon forced expression of inactive mutant NDP kinase by virtue of a dominant negative effect. NDP kinase α-transfected rat mammary adenocarcinoma cells (MTLn3) and nm23-H2-transfected human oral squamous cell carcinoma cells (LMF4) manifested reduced metastatic potential and were associated with an altered sensitivity to environmental factors, such as motility and growth factors. NDP kinase α, compared to NDP kinase β, was involved in a wide variety of the cellular phenomena examined. Taken together, NDP kinase isoforms appear to elicit both their own respective and common effects. They may have an ability to lead cells to both proliferative and differentiated states by modulating responsiveness to environmental factors, but their fate seems to depend on their surrounding milieu.

KEY WORDS: NDP kinase; nm23; isoforms; differentiation; cell growth; metastasis; signal transduction.

INTRODUCTION

Nucleoside diphosphate (NDP) kinase is a ubiquitous family enzyme involved in the high-energy transfer necessary to produce nucleoside triphosphates pools at the expense of ATP (Parks and Agarwal, 1973). Recent studies have revealed an identity between NDP kinase and nm23, Awd, PuF, and I-factor. The latter two proteins require no nucleotide phosphotransferase activity to exert their actions. Further, protein kinase activity has been reported in the NDP kinase molecules themselves. Therefore, NDP kinase is a multifunctional protein that presumably carries out more regulatory functions in the cell than ever considered. In this short review, we describe the role of NDP kinases from the perspective of cell growth, differentiation, and metastasis suppression in relation to signal transduction and discuss the implications of these functions. We use here the isoform designations, rat NDP kinase α and NDP kinase β, which

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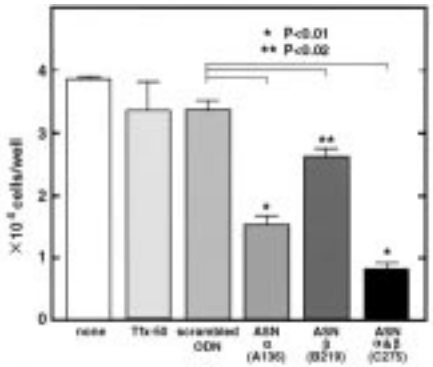
are homologs of the human NDP kinase-B/nm23-H2 and NDP kinase-A/nm23-H1, respectively.

ROLE OF NDP KINASE IN CELL GROWTH

Enhanced NDP kinase/nm23 gene expression often takes place in conjunction with enhanced cell growth (Creanor and Mitchinson, 1989; Keim *et al.*, 1992; Caligo *et al.*, 1995; Igawa *et al.*, 1994). This is consistent with other lines of evidence indicating that cell division is inhibited by microinjection of an anti-nm23 antibody into cultured cells (Sorscher *et al.*, 1993) and that cell growth is partly suppressed by treatment with antisense oligonucleotides or antisense mRNA of nm23-H1 (Cipollini *et al.*, 1997). On the other hand, when human tissues are examined for the expression of NDP kinase during the tumorigenic process, the tumor area frequently provides a stronger immunohistochemical signal than the surrounding normal area. This enhanced expression seems to occur during early phases of the tumorigenic process, since the increased mRNA and protein levels can be seen upon immortalization of human normal diploid fibroblasts by SV40 large T-antigen treatment or ^{60}Co irradiation (Ohneda *et al.*, 1994). All these observations implicate the significance of NDP kinase in cell growth and tumorigenesis. We, therefore, extended this study to determine which isoform of NDP kinase is responsible for or more associated with the proliferative activity of the cell.

To this end, we designed antisense oligonucleotides (ODN) corresponding to conserved and nonconserved nucleotide sequences of rat NDP kinase α and β isoforms, introduced these ODNs into UMR106 cells by lipofection using Tfx-50, and then examined these effects on the number of cells counted after 2 days of culture (Fig. 1). In UMR106 cells, NDPK α and β isoforms constituted 0.26 and 0.08% (shown in ng/μg protein in Fig. 1) of the total amount of cellular proteins, respectively. The effects of NDP kinase α-specific antisense ODN (A136) and NDP kinase β-specific antisense ODN (B219) were not complete, but relatively specific, resulting in a significant diminution of the corresponding protein levels. An antisense ODN (C275) designed to suppress the levels of both isoforms exerted the expected effects. Under this condition, A136 ODN and B219 ODN caused an approximately 50 and 20% suppression of the cell number, respectively, compared to the control scrambled ODN. C275 ODN produced more profound inhibition than did



	Protein levels (%)						ng / μg of total protein
NDPK α	100	121	88.7	50.8	117	63.4	2.6 ± 0.1
NDPK β	100	92.0	82.0	83.9	67.9	58.8	0.8 ± 0.1

Fig. 1. Suppression of cell growth by antisense oligonucleotides. UMR 106 cells were inoculated at a density of 3×10^3 cells/well and cultured for 27 h. Then, either a scrambled oligodeoxynucleotide (ODN), used as a control, antisense ODN specific for NDP kinase α (ASNα/A136), antisense ODN specific for NDP kinase β (ASNβ/B219), or antisense ODN common for both isoforms (ASNα & β/C275) at a final concentration of 2 μ*M* premixed with Tfx-50 (final concentration was at 3 μ*M*) in Eagle's MEM containing 10% FBS was added for 30 min at 37°C, followed by the further addition of the medium and incubation. After 24-h treatment, the medium was changed to a new one without ODNs and cultured for 1 more day. Cells were collected by trypsinization and one part of these cells was subjected to cell counting. The remainder of the cells were solubilized in 0.25 *M* sucrose–10 m*M* Tris–HCl–2 m*M* MgCl2–1 m*M* EDTA, pH 7.4 containing 10 m*M* CHAPS and then used for enzyme assay and NDP kinase isoforms protein determination using dot blot analysis with NDP kinase isoform-specific monoclonal antibodies, followed by densitometric analysis. The suppression of cell growth by antisense ODNs was apparent after 1 day of treatment; this suppression continued for at least the 3 days examined. NDPK, NDP kinase.

A136 and B219. These results suggest that the α isoform is more involved in the proliferative activity of the cell than the β isoform. However, that the reduction of both isoforms induced by C275 leads to a further suppression of cell number implies that both isoforms may play independent roles in the proliferative activity of the cell.

ROLE OF NDP KINASE IN DIFFERENTIATION

The positive role of NDP kinase during the development and differentiation of cells and tissues has also been frequently described in the literature (Lakso *et al.*, 1992; Yasutomo *et al.*, 1998). We first demon-

strated the high level of NDP kinase expression at the mRNA and protein levels in postmitotic tissues, such as heart and brain (Kimura *et al.*, 1990; Shimada *et al.*, 1993). Since then, we have been examining the role of NDP kinase during the process of development and differentiation using several cell model systems.

In bone cells, growth- and differentiation-associated genes have been studied during progressive development (Stein and Lian, 1993). In the proliferative phase of osteoblasts, growth-related genes such as c-*fos* and c-*myc* are induced, followed by the formation of an extracellular matrix during which genes such as type I collagen and fibronectin are expressed. In the subsequent period of extracellular matrix maturation, the growth-associated genes are down-regulated and proteins associated with the bone-cell phenotype (alkaline phosphatase, osteopontin, and osteocalcin) are accumulated, which contributes to mineralization. The expression of NDP kinase has been found to be low in the proliferative osteoprogenitor cells of the fetal rat calvaria, but is increased in osteoblasts actively involved in mineralization *in vivo* when analyzed by immunohistochemistry and *in situ* hybridization (Yasutomo *et al.*, 1998). In keeping with these histological observations, NDP kinase protein levels assessed by immunohistochemical analysis in cultured osteoblasts from 20-day-old rat fetal calvaria increased during differentiation in parallel with alkaline phosphatase activity in the nodules, where the cells became highly confluent and differentiated into mature osteoblasts. Interestingly, a significant amount of NDP kinase was excreted from these confluent cells into the extracellular space and accumulated in the matrix vesicles (MV), which play a role in mineralization during bone–tissue formation (Yasutomo *et al.*, 1998). It follows that NDP kinase gene expression appears to be closely related to the differentiated functions of osteoblasts and to be under the regulation of physiological regulators, such as bone morphogenetic protein, that determine osteoblast differentiation. Although the full physiological relevance of NDP kinase in osteoblast differentiation and bone formation remains to be determined, the accumulation of NDP kinase in the MV in parallel with alkaline phosphatase and some other phosphate-metabolizing enzymes (Ali *et al.*, 1970) may suggest that they work in concert for mineralization through transport and/or metabolism of calcium and phosphate.

NDP kinase α, a major form occurring in the rat, is ubiquitously expressed in most tissues, whereas NDP kinase β is abundantly present in a small number of tissues, such as the brain and testis (Shimada *et al.*, 1993). These observations have led us to speculate that NDP kinase β may play a distinct role in neuronal cells. To understand this problem, we employed a rat pheochromocytoma-derived cell line PC12D. These cells, upon the addition of differentiation inducers such as nerve growth factor (NGF) and a cyclic AMP analog (dibutyryl cyclic AMP (DBcAMP)), undergo differentiation by extruding neurites from the cell body. We transfected these cells with hemagglutinin (HA)-tagged NDP kinase α, NDP kinase β, and their mutant forms to distinguish them from the endogenous NDP kinases, from which stably transfected clones were isolated for further studies (Ishijima *et al.*, 1999). To our surprise, it was found that PC12D cell clones overexpressing HA-NDP kinase β obviously become capable of extending neurites without any differentiation inducers and show a prolonged doubling time. This effect appears to be dependent on its enzyme activity, because the expression of a mutant NDP kinase (HA-NDP kinase β H118A) for which the enzyme activity has been inactivated has not been observed to reproduce this phenotypic change. Since the overexpression of HA-NDP kinase α shows only a marginal effect, a specific role for NDP kinase β in neuronal differentiation is strongly suggested.

How is NDP kinase involved in the neurite outgrowth of PC12D cells? We have asked whether phosphotransferase activity is also required for the neurite outgrowth triggered by differentiation inducers. While cell clones overexpressing HA-NDP kinase α or HA-NDP kinase β extend neurites in response to NGF and DBcAMP, similar to the control clones, the neurite outgrowth of transfectants expressing inactive NDP kinase, HA-NDP kinase α H118A, or HA-NDP kinase β H118A, is remarkably decreased (Ishijima *et al.*, 1999). It should be noted that the NDP kinase enzyme activity present in these cell extracts is not significantly affected by transfecting active or inactive NDP kinases. Therefore, suppression by these inactive enzymes is not likely due to the altered NDP kinase enzyme activity in the cell but to their dominant negative effects. These results suggest that NDP kinase is required to interact with an unidentified target protein located along these signaling pathways and exerts its enzyme activity during the interaction (Fig. 2). Inactive NDP kinases presumably displace the endogenous enzymes from their binding site, which, as a result, leads to incomplete neurite outgrowth of PC12D cells. What is the target protein of NDP kinase? NGF and cyclic AMP are thought to share some part of their signaling pathway for PC12 cell differentiation and thus the

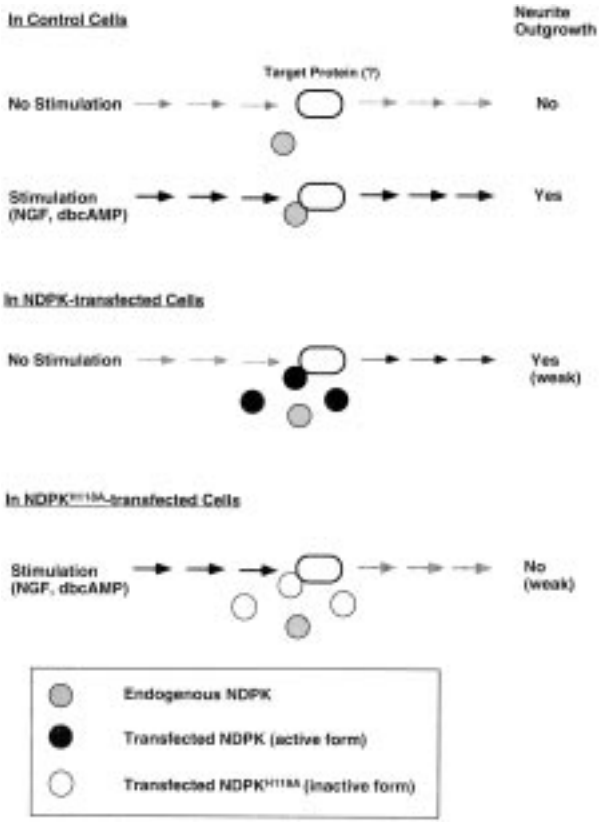


Fig. 2. Possible role of NDP kinase in PC12D cells. Upon stimulation by NGF and DBcAMP, NDP kinase (NDPK) interacts with an unidentified target protein(s) located in these signaling pathways and exerts its enzyme activity during the interaction. When a large amount of NDP kinase β is present, PC12D cells no longer require such external signals for neurite extension. Inactive mutant NDP kinases expressed in a cell presumably compete with the endogenous enzymes at their binding site and, as a result, leads to the suppression of neurite outgrowth induced by differentiation inducers.

interaction site of NDP kinases may be located in the common part of these signaling pathways. Identification of the target protein(s) of NDP kinases is under current investigation.

ROLE OF NDP KINASE/NM23 IN TUMOR METASTASIS

One of the intriguing biological features of NDP kinase/nm23 is its metastasis-suppressor activity, which was discovered by Steeg *et al.* (Liotta *et al.*, 1991; Steeg *et al.*, 1988). Thereafter, the antimetastatic activity of the NDP kinase/nm23 gene products has been established for a small panel of human and rodent tumor cell lines (Leone *et al.*, 1991, 1993; Baba *et al.*, 1995; Fukuda *et al.*, 1996; Miyazaki *et al.*, 1999) using animal model systems. However, there still remain a number of important questions that should be answered. These include: what is the intracellular mechanism by which NDP kinase/nm23 suppresses the metastatic ability of tumor cells? Which step of the metastatic process from the primary site to the distant metastatic site is affected? Why is the NDP kinase/nm23 gene expression down-regulated during tumor progression? Which isoform is responsible for the anti-metastatic action?

We have challenged some of these problems using two different cancer cell types, a rat mammary adenocarcinoma cell line (MTLn3) and a human oral squamous cell carcinoma cell line (LMF4). It has been found that when the NDP kinase α gene is transfected into a highly metastatic MTLn3 cell line, the transfected clones display diminished pulmonary metastatic potential as evaluated in a spontaneous metastasis assay, with no essential changes in a lymph node metastasis (Fukuda *et al.*, 1996). The decreased metastatic potential of these clones has also been demonstrated in an experimental metastasis assay (Fukuda *et al.*, unpublished observation). These observations strongly suggest that the anti-metastatic ability of NDP kinase α is elicited during the latter part of the metastatic process, presumably between extravasation and secondary tumor formation. Interestingly, NDP kinase β-transfected clones show only marginal effect, if any at all, under these conditions, although the transfected gene is strongly expressed. Therefore, NDP kinase α may play a more crucial role in determining the metastatic phenotype of MTLn3 than NDP kinase β.

In order to identify which step of the metastatic process is altered, we have further examined NDP kinase α- and NDP kinase β-transfected MTLn3 clones side by side in terms of presumed cell properties correlated with the altered metastatic phenotype. It was found that matrix metalloproteinases (MMPs: 80 and 60K) assessed by a gelatin zymogram assay and their adhesion potential to endothelial cells or the extracellular matrix seem unlikely to be involved in this phenomenon, as the effects of NDP kinase α and NDP kinase β genes on these parameters did not parallel those based on metastatic potential. In contrast, when the cell motility across a Transwell membrane in response to the rat lung extract is measured, NDP kinase α-transfected clones show a reduced motility compared to the control and NDP kinase β-transfected clones (Fukuda *et al.*, unpublished observation). Thus, cell motility appears to be a candidate step that is affected by NDP kinase/nm23 during the metastatic process.

Highly metastatic human oral squamous cell carcinoma (SCC) cells show a reduced expression of nm23-H2/NDP kinase B compared to low and nonmetastatic cells (Miyazaki *et al.*, 1999). In addition, no clearly altered expression of nm23-H1/NDP kinase-A has been detected, at least at the protein level. We, therefore, introduced the *nm23-H2/NDP kinase-B* gene into one of these metastatic human SCC cells (LMF4) and isolated highly expressed clones (H2-5 and H2-7). These clones were found to be less metastatic when they were iv-injected into nude mice and the lung metastasis was examined 30–40 days later (Miyazaki *et al.*, 1999). Consequently, nm23-H2/NDP kinase-B appears to have the ability to suppress metastasis of a certain human cancer cell. The uncertainty regarding the antimetastatic action of nm23-H1 in LMF4 cells can primarily be ascribed to the failure to obtain high-expression clones.

The nm23-H2/NDP kinase-B-transfected LMF4 clones display a number of intriguing features (Miyazaki *et al.*, 1999). They show a higher growth rate in DMEM 10% fetal bovine serum (FBS) than the control clones—doubling time being shortened from 47 to 28 h. The transfected clones acquire the ability to multiply at a low serum concentration (0.6%) and become unresponsive to growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and insulin. However, they increase their cell number in response to transferrin, as did the control clones in the above study. These features were unexpected, since previous studies had documented that the antimetastatic action of NDP kinase/nm23 occurs independently of the proliferative activity in culture and of the primary tumor size of the transfected cells. It should be emphasized that both H2-5 and H2-7 clones exhibit differentiated morphology at the metastatic sites (in the lung) after iv inoculation into nude mice. Therefore, the effect of nm23-H2/NDP kinase-B is paradoxical as it appears to be growth-promoting under culture conditions, but differentiation-promoting in certain tissues *in vivo*. It is conceivable that nm23/NDP kinase suppresses the metastatic propensity of LMF4 cell as a result of modulating growth and/or differentiation potential in response to environmental factors.

ROLE OF NDP KINASE IN SIGNAL TRANSDUCTION

We originally proposed that NDP kinase may act to supply GTP for the heterotrimeric G proteins, which absolutely require GTP for their own activation (Kimura, 1993). The basis for this proposal is that NDP kinase allows rapid and effective activation of GTP binding (G) proteins and, in turn, effectors. In bovine retinal rod outer segment (ROS) preparations it has been found that NDP kinase exhibits an equilibrium binding to the bovine retinal ROS membranes via the G protein transducin (Gt) (Orlov *et al.*, 1996). Further, we have provided experimental evidence that NDP kinase may have the potential to modulate a number of other signal-transduction systems, including tyrosine kinase signalings (e.g., NGF, PDGF) (Ishijima *et al.*, 1999; Miyazaki *et al.*, 1999). In fact, there have been reports that NDP kinase/nm23-transfected clones accompany diminished sensitivity to transforming growth factor-β (TGF-β) in soft agar colonization (Leone *et al.*, 1991, 1993), reduced cell motility in response to IGF-1, PDGF, and serum (Kantor *et al.*, 1993), and enhanced formation of basement membrane and growth arrest when cultured in reconstituted basement membrane components (Howlett *et al.*, 1994).

FUNCTIONAL COMPARISON BETWEEN NDP KINASE ISOFORMS

In higher animals there are two major NDP kinase isoforms that are highly homologous and form a mixed hexameric complex in the cell (Shimada *et al.*, 1993). These isoforms show different tissue distribution and intracellular localization; as a result, multiple functions of NDP kinase could be ascribed to the differential behavior of the isoforms. The monomeric subunits differ in 16 out of 152 amino acids; 7 and 3 of these amino acid substitutions are clustered in two regions, one near the N-terminal third (V1 region) and the other at the C-terminal end (V2 region), respectively (Fig. 3). Crystallographic studies (Moréra *et al.*, 1995) have revealed that the V1 region encompasses the region from the β2 strand to the αA helix and extends to the outer surface of the hexameric structure of both isoforms. This arrangement implies that if NDP kinase exerts its action through interactions with other proteins in an isoform-dependent manner, both the V1 and V2 regions appear to be the most probable candidate sequences responsible for such interactions. Indeed, the binding of NDP kinase to the ROS membranes, which occurs in a G protein (Gt)-dependent manner, is interrupted by a peptide corresponding to the V1 region of NDP kinase α, not NDP kinase β (Orlov *et al.*, 1996).

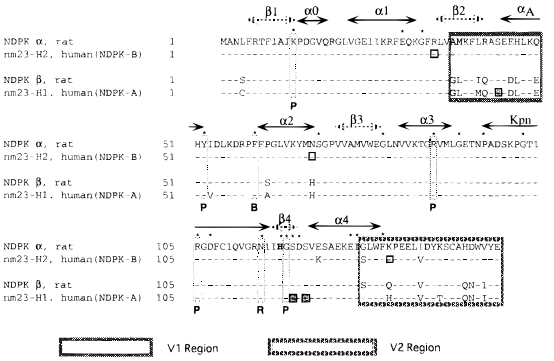


Fig. 3. Primary and secondary structures of NDP kinase isoforms. Two major rat and human NDP kinase (NDPK) isoforms are shown for comparison. His118 is the active site that forms the phosphorylated intermediate; amino acids with asterisks denote those that are completely conserved from bacteria to mammals; amino acids boxed with dotted lines and the capital letters B, P, and R represent those that interact with the base, phosphate, and ribose of the substrate, respectively; three amino acids with open squares are responsible for DNA binding; serine residues with shaded squares are phosphorylation sites; V1 and V2 regions are those enriched with nonconserved amino acids between the two isoforms (and among various species).

The experimental data described above implicate that NDP kinase isoforms seem to have their own, as well as common, functions in a variety of phenomena (Table I). At a glance, NDP kinase α is associated with a wide range of the cellular phenomena so far examined. In contrast, NDP kinase β is likely to exhibit its influence in very limited cellular functions. These results support the previous notion (Shimada *et al.*, 1993) that NDP kinase α plays a larger and more general role in most cells than NDP kinase β, at least in rat.

POSSIBLE DUAL ROLE OF NDP KINASE IN CELLULAR FUNCTIONS

Considering the housekeeping nature of NDP kinases, it is plausible that there is a mechanism that keeps NDP kinase protein levels constant to maintain their function in a variety of cells (Ishikawa *et al.*, 1997). In fact, this has been illustrated in a number of experiments in which NDP kinase protein levels have been more stable in response to various exogenous stimuli than their mRNA levels. A recent discovery of multiple transcripts for rat NDP kinase with different translation efficiencies (Ishikawa *et al.*, 1997) may partly explain such a discrepancy between mRNA and protein levels. To further investigate this problem, we employed a tetracycline-controlled expression system allowing the expression of exogenously introduced NDP kinase in a tetracycline-dependent manner in a cell and attempted to determine whether endogenous NDP kinase protein levels are affected by the expression of exogenous NDP kinase. The results demonstrated that the endogenous NDP kinase levels are suppressed by the coexpression of exogenous NDP kinase (Ishikawa *et al.*, unpublished observation), implying the presence of a negative-feedback regulation of NDP kinase genes by their protein products.

Table I Functional Comparison of NDP Kinase/nm23 Isoforms

Functional involvement	NDP kinase α (nm23-H2)	NDP kinase β (nm23-H1)[a]
Growth-promoting (supporting) action for		
UMR106	Yes	Weak
LMF4 (in culture)	Yes	ND
PC12D	No	Yes (Suppressive)
Differentiation-promoting action for		
PC12D	Weak	Yes
LMF4 (*in vivo*)	Yes	? (No)
Anti-metastatic action for		
MTLn 3	Yes	No
LMF4	Yes	? (No)
Signal transduction		
Interaction with ROS (Gt-dependent)	High affinity	Low affinity
Neurite outgrowth (PC12D) by NGF and DBcAMP	Yes	Yes
Growth stimulation (LMF4) by PDGF, IGF-1, and insulin	Yes (Suppressive)	ND

[a] ND, not determined; ?(No), the effect is not clear because of the low expression of nm23-H1.

Although important questions as to the feedback regulation mechanism, isoform specificity, and requirement for enzyme catalytic activity remain to be delineated, NDP kinase genes appear to be under the regulation of their own products. It has been proposed that human NDP kinase-B may act as a transcription factor (Postel *et al.*, 1993). What then happens when NDP kinase levels are drastically perturbed? The data obtained thus far suggest that altered levels of NDP kinase protein beyond a certain range can lead to drastic changes in cell phenotypes. Notably, NDP kinase/nm23 appears to elicit a dual action by virtue of, most probably, modulation of a responsiveness to environmental factors; one of these actions leads to cell growth potentiation and the other contributes to cell development and differentiation. Moreover, as shown in the transfected LMF4 clones (Miyazaki *et al.*, 1999), such a bidirectional potentiation seems to occur in a single cell type, depending on the surrounding environment.

CONCLUDING REMARKS

In this study we have emphasized the importance of comparing NDP kinase isoforms side by side to delineate their overall relationship to a wide variety of phenomena, since they exhibit individual, as well as common, functions in the cell. This approach should help us to understand the multiple roles of NDP kinases. In addition, to expedite our understanding of the enigmatic characteristics of NDP kinases, we hypothesize that the phenotypic change induced by exogenous NDP kinase may become bidirectional depending on the environmental conditions under which a given cell is investigated.

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