NM23/Nucleoside Diphosphate Kinase and Signal Transduction

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NM23s (or NDP kinases) regulate a fascinating variety of cellular activities, including proliferation, development, and differentiation. All these processes are modulated by external stimuli, leading to the idea that this family of proteins modulates transmembrane signaling pathways. This review summarizes the evidence indicating that NM23/NDP kinases participate in transmembrane signaling in eukaryotic cells and discusses the molecular mechanisms proposed to account for these actions.

INTRODUCTION

NM23/NDP kinases constitute a family of proteins with key roles in the regulation of normal cell function. Traditionally, NM23/NDP kinases were ascribed a single physiological function, namely the synthesis of nucleoside triphosphates, other than ATP, through their nucleoside diphosphate kinase (NDP kinase) activity (Parks and Agarwal, 1973), but an increasing number of observations implies that these proteins are multifunctional.

Two of the NM23/NDP kinase isoforms, 1 and 2, are widely expressed in large amounts and have been extensively studied. These highly homologous proteins are present in cells at concentrations in the micromolar range and account for the bulk of NDP kinase activity in cells. The recent finding that there are multiple NM23/NDP kinase gene products in most cells (Lacombe *et al.*, this issue) raises the question as to why are there so many NM23/NDP kinases, given the existence of two isoforms that are amply expressed and very active as enzymes. It is not known whether different NM23/NDP kinases carry out unique or redundant cellular functions, but on the basis of the overlapping individual patterns of tissue expression of distinct isoforms, one would expect them to perform specialized functions. Moreover, primary structure analysis shows that all NM23/NDP kinases contain a set of highly conserved residues devoted to nucleotide binding and catalysis, but in different isoforms these common elements are interspersed with regions displaying significant structural diversity. In particular, the N and C terminal regions are highly variable in both length and structure prompting the speculation that the nonconserved regions of NM23/NDP kinases may be associated with functions unrelated to nucleotide phosphorylation. Indeed, the NDP kinase activity of the *Drosophila* NM23 homolog is not sufficient for its biological function (Xu *et al.*, 1996) and, furthermore, some NM23 proteins do not have NDP kinase activity (Munier *et al.*, 1998; Ogawa *et al.*, 1996), implying that NM23s are more than just NDP kinases.

Alterations in the expression levels or modifications in the structure of NM23/NDP kinases profoundly affect cell proliferation, development, and differentiation by mechanisms that are not well understood. It is noteworthy, however, that all these cellular activities are regulated by signal transduction processes, where external molecules bind to surface receptors and generate a signal that is transmitted to an

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intracellular target by a network of interacting proteins. This minireview provides a summary of the experimental evidence that led to the hypothesis that eukaryotic NM23/NDP kinases affect multiple cellular processes by regulating the propagation of signals from receptors to effectors and examines the molecular mechanisms proposed to account for this role.

CELLULAR STUDIES SUPPORT A ROLE FOR NM23/NDP KINASES IN SIGNAL TRANSDUCTION

There is a large body of observations that document the involvement of NM23/NDP kinase in the responsiveness of cells to extracellular stimuli. Thus, melanoma and breast carcinoma cells overexpressing NM23-H1 display a reduced response to the cytokine TGF-β1 (Leone *et al.*, 1991, 1993) and their motility in response to serum, PDGF, and IGF-1 is markedly inhibited (Kantor *et al.*, 1993; MacDonald *et al.*, 1996; Russell *et al.*, 1998). Similarly, NM23-H1 suppresses chemoattractant-stimulated motility of prostate cancer cells (Lee and Lee, 1999), while decreased expression of NM23-H1 through antisense ablation blocks the TGF-β1 signaling pathway leading to growth inhibition of colon carcinoma cells (Hsu *et al.*, 1994). Effects of other isoforms on signaling have also been reported. For instance, overexpression of NM23-H2 in oral carcinoma cell lines causes loss of responsiveness to PDGF, IGF-I, and insulin (Miyazaki *et al.*, 1999) and cells from normal bone marrow fail to differentiate upon treatment with GM-CSF when transfected with NM23-H3 (Venturelli *et al.*, 1995). There are inconsistent observations on the effect of NM23/NDP kinases on NGF signaling in PC12 cells: in one case, overexpression of NM23-H1 promoted NGF-induced differentiation (Gervasi *et al.*, 1996), while, in another, it caused cells to differentiate in the absence of NGF (Ishijima *et al.*, 1999).

Conversely, manipulation of NM23/NDP kinase expression levels in breast cancer cells (Otero *et al.*, 1999), as well as studies with specific antibodies in atrial myocytes (Xu *et al.*, 1996), indicate that NM23-H1 promotes sustained activity of muscarinic K^+ channels in the presence of agonist, thereby enhancing the response. Furthermore, studies in fission yeast show that a dominant negative mutant of NM23/NDP kinase reduces gene expression in response to mating pheromone signaling (Izumyia and Yamamoto, 1995). In plants, NM23/NDP kinase is a positive element of

the light-detection system that utilizes phytochromes (Choi *et al.*, 1999).

Site-directed mutagenesis of NM23-H1 also reveals discrepancies in the actions of NM23 on different signal transduction processes. Thus, in breast carcinoma cells transfected with NM23-H1 mutants, the substitutions P96S and S120G eliminate the protein's ability to suppress motility (MacDonald *et al.*, 1996), but only the S120 mutant loses the ability to enhance muscarinic K^+ channel activity (Otero *et al.*, 1999).

Evidently, NM23/NDP kinases modulate cellular signal transduction networks. However, the results do not follow an obviously discernible pattern, so that, to date, there is no unifying hypothesis for the role of NM23/NDP kinase in signal transduction. One should note, however, that the apparently conflicting results obtained in different cells, cancerous or normal, seem to depend strongly on the class of receptor being activated. Thus, in the cases where NM23/NDP kinase enhances a response, the receptors in question (muscarinic cholinergic m2 receptor, mating factor receptor, or phytochrome) are coupled to heterotrimeric G proteins, while inhibitory effects of NM23/NDP kinase are seen with cytokines and most polypeptide growth factors. Collectively, then, these reports are consistent with a pattern where NM23/NDP kinases play opposite roles in signal transduction depending on whether the extracellular signal activates a receptor that triggers heterotrimeric G protein activation or utilizes tyrosine/serine phosphorylation to initiate signals. If this model is correct, NM23/NDP kinase would be anticipated to influence nonidentical events in these two types of signaling cascades. Moreover, it explains why structural changes brought about by point mutations in NM23/NDP kinase affect G protein-coupled systems and growth factor receptor signaling in contrasting ways. Nevertheless, this speculation is based in a limited number of observations and additional experimentation with both types of receptors is required to determine its validity.

POTENTIAL MECHANISMS FOR THE PARTICIPATION OF NM23/NDP KINASES IN SIGNALING

Various mechanisms have been proposed to account for the observed effects of NM23/NDP kinases in signal transduction. Because NM23/NDP kinases have NDP kinase activity, it was hypothesized that NM23/NDP kinases might stimulate GTP-dependent

processes such as tubulin polymerization or G protein activation either by synthesizing GTP in the vicinity of microtubules or plasma membranes or, on the other hand, through direct phosphorylation of the bound GDP. Subsequently, the discovery that NM23/NDP kinases have biological activities that are not connected to nucleotide phosphorylation led to the formulation of new mechanisms for its participation in signaling. Current theories for NM23/NDP kinase's actions in cells focus on its ability to transfer phosphate into other proteins, or on its interactions with several other cellular components. The arguments and evidence supporting or opposing each of these hypotheses are briefly discussed below.

Direct Phosphorylation

Purified GTP-binding proteins show strict specificity for guanine nucleotides; nevertheless, when their activity is measured in intact cells or isolated plasma membranes, other nucleotides are often able to substitute for GTP. This effect usually originates through nucleotide interconversion mediated by the NDP kinase activity of membrane-associated NM23 (Kimura and Shimada, 1983; Otero *et al.*, 1988). This finding led to the suggestion that NM23/NDP kinases could transfer the terminal phosphate of nucleotide triphosphates directly to inactive, GDP-bound GTP-binding proteins, thus converting them into the active, GTP-bound form. The direct phosphorylation hypothesis, initially embraced with enthusiasm (Kikkawa *et al.*, 1990, Randazzo *et al.*, 1991; Ruggieri and McCormick, 1991), fell out of favor when it became clear that the protocols utilized to demonstrate direct transfer of phosphate from NM23/NDP kinase to G proteins gave rise to artifacts. Most of the technical difficulties associated with these experiments come from intrinsic properties of both GTP-binding proteins and NM23/NDP kinase. Namely, in the presence of nucleotide triphosphate any GDP released by GTP-binding proteins will be rapidly converted to GTP by NM23/NDP kinase and promptly reassociate to its specific binding site, giving the false impression that *in situ* rephosphorylation has taken place (Kikkawa *et al.*, 1991, Randazzo *et al.*, 1992). However, even when the GDP release problem is eliminated, other properties of Nm23/NDP kinase can confound the interpretation of the results. In a recent report, attempts were made to demonstrate direct phosphorylation by cross-linking GDP to the small GTP-binding protein Rad by pro-

longed UV treatment (Zhu *et al.*, 1999). Subsequent incubation of GDP-bound Rad with Nm23/NDP kinase and [γ³²P]ATP resulted in radiolabeling of Rad and this was taken as evidence of *in situ* GDP phosphorylation. However, NM23/NDP kinase is able to transfer phosphate into denatured proteins (Engel *et al.*, 1995) and might target the UV-damaged polypeptide chain of the G protein; therefore, it is not certain that the structure labeled under these conditions was the GDP. A similar approach was utilized to support the idea that NM23/NDP kinase not only phosphorylates the GDP bound to Rad, but, in addition, is a GAP, that is, increases their GTPase activity. GTP was covalently linked to Rad by UV irradiation prior to incubation with Nm23/NDP kinase (Zhu *et al.*, 1999). Unfortunately, demonstration of GAP activity required short exposures to UV light to avoid denaturation of Rad, thus reducing the yield of covalently linked GTP. Therefore, the observed increase in GTPase activity could also have been caused by release of GTP into the solution and its conversion into GDP by NM23/NDP kinase.

Structural constraints provide a compelling argument against direct phosphorylation of the GDP bound to GTP binding proteins by NM23/NDP kinase. The crystal structures of NM23/NDP kinase proteins shows that the putative phosphate donor, a phosphohistidine, is buried in a crevice on the side of the hexamer (Dumas *et al.*, 1992), whereas GDP, the target of the phosphoryl transfer, is bound in a deep cleft on GTP-binding proteins (reviewed by Sprang, 1997) and not easily accessible. In the case of the heterotrimeric G proteins, there are additional difficulties: a helical domain buries the nucleotide further and binding of the βγ dimer to G_α– GDP traps the nucleotide in its binding pocket (Sprang, 1997). Interaction between NM23/NDP kinase and inactive G proteins would have to induce dramatic changes in their conformation in order to release Gβγ and produce a suitable spatial arrangement allowing phosphate transfer. Therefore, proof of this hypothesis awaits the isolation of NM23/NDP kinase-G protein complexes and the determination of their three-dimensional structure.

GTP Channeling

NM23/NDP kinase is found in subcellular fractions or structures enriched in proteins that require GTP for activity, such as microtubules, ribosomes and plasma membranes (Kimura and Shimada, 1988; Otero *et al.*, 1988; Sonneman and Mutzel, 1995; Pinon *et*

al., 1999). This observation inspired the hypothesis that NM23/NDP kinases support the activation of GTP-binding proteins by synthesizing GTP in their immediate vicinity (reviewed in Otero, 1990), in a process akin to substrate channeling. Nevertheless, reports of channeling of nucleotides by NM23/NDP kinase are fraught with conceptual and technical problems, similar to those discussed above for direct phosphorylation. First, channeling is defined as the transfer of a metabolite between the active sites of two domains of a multifunctional enzyme or between components of a stable multienzyme complex (Miles *et al.*, 1999), but complexes between GTP-binding proteins and NM23/NDP kinase have yet to be isolated and characterized. Second, GTP-binding proteins are characterized by very high affinities for GTP (in the nanomolar range) as well as low turnover rates; hence, diffusion of intracellular GTP, which is of the order of 100 μM, appears to be sufficient to replenish the GTP hydrolyzed during signaling (Berman and Gilman, 1998). In addition, most experiments supporting a channeling mechanism were performed in preparations with poorly defined components, usually membranes or partially purified microtubules, and are, therefore, difficult to interpret. Moreover, in instances where this hypothesis was tested directly, it was found to be incorrect. For instance, localized GTP synthesis by NM23/NDP kinase is not involved in the activating effects of ATP on G protein-activated muscarinic K^+ channels (Xu *et al.*, 1996; Sorota *et al.*, 1998). In the case of tubulin, no kinetic interaction was observed between purified NM23/NDP kinases and tubulin dimers, assembled microtubules, or tubulin–microtubule-associated protein (MAP) oligomers (Melki *et al.*, 1992). Thus, to date there is no convincing kinetic or structural evidence supporting the existence of channeling between NM23/NDP kinase and GTP-binding proteins.

Protein Phosphorylation

Several laboratories have reported that NM23/NDP kinase can transfer phosphate onto other proteins, leading to the proposal that NM23/NDP kinase affects signaling through protein phosphorylation, similarly to the protein kinases that are part of phosphorylation cascades activated in response to receptor stimulation.

NM23/NDP kinase from mast cells phosphorylates serine residues in histone 2b and casein (Hemmerich and Pecht, 1992), as well as ovalbumin (Inoue *et*

al., 1996). In the presence of 1M urea, proteins in heat-denatured cell extracts are phosphorylated on serines and threonines by NM23/NDP kinases from several organisms (Engel *et al.*, 1995), although the physiological significance of this is difficult to evaluate. NM23/NDP kinases also phosphorylate histidines on the catalytic sites of ATP-citrate lyase (Wagner and Vu, 1995), succinic thiokinase (Freije *et al.*, 1997; Wagner *et al.*, 1997), and in the bacterial histidine protein kinases EnzV and CheA (Lu *et al.*, 1996). NM23/NDP kinase was also found to phosphorylate aspartates or glutamates in 43-kDa proteins from brain membranes (Wagner *et al.*, 1997). Protein phosphorylation is affected in unexpected ways by site-directed mutagenesis of NM23/NDP kinase. Whereas mutation of the catalytic site histidine invariably eliminates both NDP kinase and protein phosphotransferase activities of NM23/NDP kinase, point mutants that have only 0.1–2% of the NDP kinase activity of the wild-type protein retain 20–40% of their ability to phosphorylate proteins in the presence of urea (Engel *et al.*, 1995). Other NM23/NDP kinase mutants that retain full NDP kinase activity show slightly diminished phosphotransferase activity toward histidines, but are unable to phosphorylate acidic residues (Wagner *et al.*, 1997).

However, the hypothesis that NM23/NDP kinase modulates signal transduction through the phosphorylation of other proteins has to be taken with caution, in view of the atypical features of the phosphotransferase activity of NM23/NDP kinases. Namely, protein phosphorylation by NM23/NDP kinase is a slow process with half times of the order of minutes, requires not catalytic but stoichiometric amounts of enzyme and targets assorted types of proteins and amino acid side chains seemingly without specificity. In contrast, eukaryotic transmembrane signaling cascades rely on rapid changes in protein phosphorylation through the regulated activity of protein kinases that act catalytically on a very restricted set of proteins and show specificity for serine/threonines and/or tyrosines. Nevertheless, the natural protein substrates for NM23/NDP kinases may not have been identified yet and it is thus possible that eventual discovery of a physiologically relevant target may reveal a genuine protein kinase activity of NM23/NDP kinase.

Protein–Protein Interactions

To understand the function of a protein, it is helpful to identify other proteins with which it associates,

either by biochemical or genetic methods. There are two reports of a direct interaction of NM23/NDP kinase with signaling proteins. In both cases this association was revealed during a yeast two-hybrid screen for novel receptor binding partners; the interaction was verified by an independent technique. The two-hybrid approach led to the discovery that NM23/NDP kinase from *Arabidopsis* associates directly with the activated form of the far-red phytochrome receptor (Choi *et al.*, 1999) and that mammalian NM23/NDP kinase 1 and 2 interact with nuclear orphan receptors, a group of structurally related transcription factors that regulate target gene transcription in response to unknown ligands (Paravicini *et al.*, 1996). Interestingly, neither phytochrome nor nuclear orphan receptors are transmembrane proteins, as are the growth factor or G protein-coupled receptors. Rather, these are receptors that localize to the cytoplasm and to the nucleus, playing a key role in intracellular signaling. Interactions between NM23/NDP kinase and other bona fide signaling proteins have yet to be uncovered, maybe because the complexes involved rely on weak or transient interactions, as seen often in signal transduction processes.

On the other hand, stable complexes between NM23/NDP kinases and a number of different cellular ligands have been isolated and some of these proteins could possibly provide a physical link between NM23/NDP kinase and transmembrane signaling cascades. In higher organisms the binding partners identified for NM23/NDP kinases include vimentin, either in soluble form or assembled into intermediate filaments (Otero, 1997; Pinon *et al.*, 1999), the antioxidant protein AOP1 (Otero, 1997), molecular chaperones of the Hsp70 family (Leung and Hightower, 1997; Barthel and Walker, 1999), enzymes involved in DNA precursor biosynthesis (Harvey and Pearson, 1988), and several enzymes involved in energy metabolism, such as ATP-citrate lyase, succinate thiokinase, creatine kinase, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase (Kadrmas *et al.*, 1991; Wagner and Vu, 1995, Otero, 1997). In addition, NM23/NDP kinase 1 and 2 colocalize with microtubules in cells (Pinon *et al.*, 1999) and in cell extracts (Lombardi *et al.*, 1995); binding to tubulin is probably indirect, being mediated by other, intermediary proteins. Indeed, some of the proteins that associate with NM23/NDP kinase, such as HSC70, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase are known to interact with microtubules (Liang and McRae, 1997; Janmey, 1998).

The proteins that are most likely to link NM23/NDP kinase to signaling processes are the cytoskeletal components, tubulin and vimentin, which are involved in the spatial features of cell signaling. Cytoskeletal interactions of NM23/NDP kinase have been observed in cells from a variety of organisms and tissues; the ubiquity of this association suggests that it serves an essential function (Biggs *et al.*, 1990; Otero, 1997; Pinon *et al.*, 1999). Intermediate filaments and microtubules bind numerous signaling elements such as protein kinases, phosphatases, lipid kinases, phospholipases, and several GTPases (reviewed by Janmey, 1998) and both tubulin and vimentin are found in macromolecular complexes formed following stimulation by extracellular factors (Yeung *et al.*, 1998; Liao *et al.*, 1999; Schmid-Alliana *et al.*, 1998). Therefore, it is possible that, as seen with other signal transduction molecules, association with the cytoskeleton mainly mediates the recruitment of NM23/NDP kinase to multimeric signaling complexes that arise in response to activation of transmembrane receptors.

On the other hand, the cytoskeleton could be not a localizing device for NM23/NDP kinase, but instead one of its targets. Namely, activation of transmembrane receptors often affects cytoskeletal organization and the association of NM23/NDP kinase with structural proteins, such as vimentin, could affect the dynamics of cytoskeleton remodeling following external stimulation. Vimentin networks show a tendency to form tangles and bundles and be more densely packed when assembled in the presence of NM23/NDP kinase, presumably because more than one filament can interact with the NM23/NDP kinase hexamer (Fig. 1). If NM23/NDP kinase has such an effect *in vivo*, its overexpression could reduce the flexibility necessary for cytoskeleton plasticity and cell movement (Eckes *et al.*, 1998; Gilles *et al.*, 1999) and this property could be the basis for the motility supressive actions of some NM23/NDP kinase isoforms. This model predicts that destabilization of the hexameric structure of NM23/NDP kinase will have a negative impact on function. Indeed, naturally occurring mutants of NM23/NDP kinase associated with abnormal phenotypes (P96S, Lascu *et al.*, 1992; S122P, Hamby *et al.*, 1995; S120G, Chang *et al.*, 1996) retain considerable catalytic activity but are either intrinsically unstable (Schaertl *et al.*, 1999) or show increased susceptibility to denaturation by heat and urea (Chang *et al.*, 1996; Lascu *et al.*, 1992, 1997). One of these mutations increases the binding of a protein from HL-60 cells to NM23/NDP kinase (Chang *et al.*, 1996), suggesting that destabilization of the structure can indeed be accompanied by
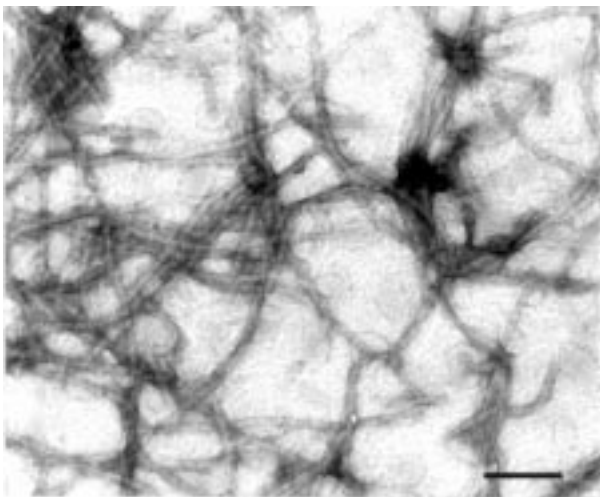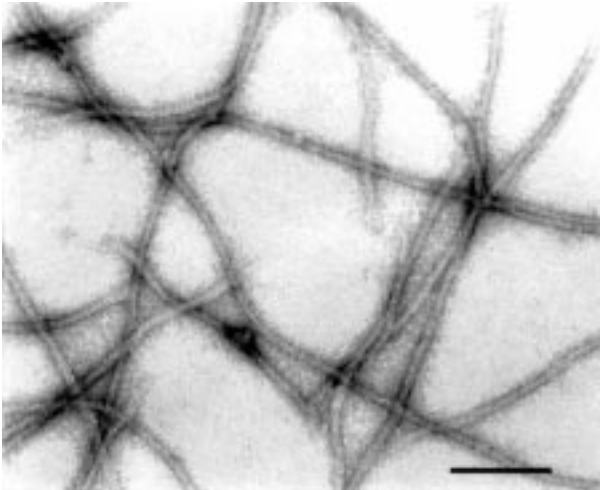
Fig. 1. Effect of increasing concentrations of NM23/NDP kinase on assembly of vimentin intermediate filaments. Recombinant hamster vimentin (0.5 mg/ml) was combined with 0.005 mg/ml of bovine serum albumin (left panel) or purified frog NM23/NDP kinase (right panel) in 5 m*M* HEPES, pH 7.0, with 1 m*M* DTT for 30 min at 22°C. Assembly was then induced by adding NaCl to 150 m*M*. After 30 min at 35°C, samples were deposed in grids and negatively stained with uranyl acetate. Bar: 100 nm.

alterations in the specificity of protein–protein association.

Whatever the case may be, knowledge of the relationship between NM23/NDP kinase function and its association with structural proteins is instrumental if we are to understand the many effects of this family of proteins in cell function.

CONCLUSIONS

Considerable advances have been made in identifying novel cellular functions of NM23/NDP kinases.

However, notwithstanding the numerous studies implicating the NM23/NDP kinase family in signaling, many questions remain unsolved, in particular, regarding the role of NM23/NDP kinases in signal transduction, since the ubiquitous tissue expression of the most abundant isoforms has made it difficult to precisely determine their role in a neutral background. Recent experiments employing knockout technologies will certainly help establish the significance of NM23/NDP kinases to cell function and will serve as a valuable tool to differentiate the physiological functions of these proteins.

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