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Protein Interactions Provide New Insight Into Nm23/Nucleoside Diphosphate Kinase Functions

D. Lombardi[1,2,4] and A. M. Mileo[3]

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Nm23–NDPKs besides contributing to the maintenance of the cellular nucleoside triphosphate pool, exert regulatory properties in a variety of cellular events including proliferation, invasiveness, development, differentiation, and gene regulation. This review focuses on recently discovered protein–protein interactions involving the Nm23 proteins. The findings herein summarized provide new and intriguing suggestions for a more extensive understanding of the biological functions of the Nm23 proteins.

KEY WORDS: Nm23; nucleoside diphosphate kinase; metastasis; differentiation; transcription; protein–protein interaction; two-hybrid system.

INTRODUCTION

The first *nm23* cDNA was identified, by subtractive cloning, in murine melanoma cell lines endowed with different metastatic potential (Steeg *et al*., 1988). Since then, a second murine (Urano *et al*., 1992) and eight human (*nm23-H1* to *nm23-H8*) (reviewed in Lacombe *et al*., 2000) genes were identified.

The *nm23-H1* and *nm23-H2* gene products were recognised as nucleoside diphosphate kinases (NDPK) A and B, respectively (Gilles *et al*., 1991). Moreover, NDPK activity was also reported for the murine Nm23 proteins (Urano *et al*., 1992) and the majority of the human Nm23 proteins (reviewed in Lacombe *et al*., 2000). NDPKs are ubiquitous enzymes that catalyze the phosphorylation of nucleoside diphosphates to nucleoside triphosphates at the expense of ATP (Parks and Agarwal, 1973).

[1]Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy.
[2]Regina Elena Cancer Institute, Centre for Experimental Research, Roma, Italy.
[3]Laboratory "C", Department for the Development of Therapeutic Programs, Regina Elena Cancer Institute, Centre for Experimental Research, Roma, Italy.
[4]To whom correspondence should be addressed at Regina Elena Cancer Institute, Centre for Experimental Research, Via delle Messi d'Oro, 156, 00158 Roma, Italy; e-mail: lombardi@ifo.it.

The existence of multiple Nm23 proteins rises the question whether they carry out redundant NDPK activity or, instead, perform specialized functions, not necessarily related to nucleoside phosphorylation, and probably depending on different cellular contexts. Extensive studies demonstrated that the Nm23 proteins participate in the regulation of a broad spectrum of cellular functions such as, signal transduction (reviewed in Otero, 2000), development and differentiation (reviewed in Lombardi *et al*., 2000), and gene expression (reviewed in Postel *et al*., 2000). It was largely documented that Nm23-H1 and -M1 suppress the invasive phenotype (reviewed in De La Rosa *et al*., 1995 and in Lombardi *et al*., 2000). Moreover, Nm23-H1 and -M1 promote the onset and contribute to the maintenance of the neural differentiated phenotype by interplaying with negative regulators of the cell cycle (Gervasi *et al*., 1996; Lombardi *et al*., 2001). Nm23-H2 regulates transcription of different genes by its ability to recognise and alter structural elements of DNA (reviewed in Postel *et al*., 2000). Interestingly, the enzymatic activity is not required for metastasis suppression (MacDonald *et al*., 1993), for the control of the differentiated phenotype (Lombardi *et al*., 2001) and for gene transcription (reviewed in Postel *et al*., 2000).

The molecular mechanisms underlying the role of the Nm23 proteins in the different cellular processes are still object of extensive studies.

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PROTEIN–PROTEIN INTERACTIONS

Since proteins that are found in the same complex or in the same location are likely to be involved in the same or related cellular process, a useful approach to unravel the function of a protein is to identify other proteins, possibly of known functions, with which it interacts. Protein biochemistry or genetic methods can reveal protein–protein interactions.

Reports based on canonical protein biochemistry approaches indicated that Nm23 proteins could be found associated with components of the cytoskeleton network such as tubulin (Lombardi *et al*., 1995; Pinon *et al*., 1999) and vimentin (Otero, 1997). The association with tubulin, observed in cells of different origin, was modulated during essential processes as cell invasion and differentiation (Lombardi *et al*., 1995), presumably affecting cytoskeleton remodelling. The findings might indicate a direct involvement of Nm23 in such phenomena or, alternatively, a recruitment of the protein to pathways regulating cell shape and/or cell motility. Therefore, specific functions of the Nm23 proteins in the cytoskeleton machinery are not elucidated.

Nm23-H1 was reported to physically interact with Rad and suggested to act as a bifunctional regulator of the Ras-related GTPases, promoting both GTP hydrolysis and GTP reloading (Zhu *et al*., 1999). Rad is overexpressed in skeletal muscle from type 2 diabetes patients (Reynet and Kahan, 1993) and causes a marked reduction in glucose uptake in response to insulin, suggesting that it contributes to diabetes insulin resistance (Moyers *et al*., 1996). In this scenario, it is unclear the role of the association, actually not convincingly proved, between the two proteins.

Moreover, Nm23-H1 was found to interact with Tiaml (Otsuki *et al*., 2001), the specific nucleotide exchange factor for Racl, a Rho-family GTPase involved in the induction of metastases (Habets *et al*., 1994). The interaction was detected by coimmunoprecipitation from cells expressing exogenous Nm23-H1 and Tiaml and from mouse brain extracts. Rac1 and Tiaml are involved in the formation of ruffles and lamellipodia (Sander *et al*., 1999). Nm23-H1 inhibited the Tiaml activation of Rac1 and, upon overexpression, inhibited ruffle and lamellipodium formation. The inhibition of the Tiaml–Racl pathway by Nm23-H1 fits well with the metastasis suppressor role of the NDPK. Anyway, since the Authors could not demonstrate a direct interaction between recombinant Nm23-H1 and Tiaml proteins, it is possible that the association may require other linking protein(s).

More arguments to the disclosure of the functions of the Nm23/NDPKs derive from the individuation of protein interactions via a genetic approach. This review will provide a rapid excursus throughout a series of experimental results obtained by mean of the yeast two-hybrid system (Fields and Song, 1989), a genetic assay based on the properties of site-specific transcriptional activators.

Nm23-M1 as Bait in the Yeast Two-Hybrid System

An attempt to individuate protein interactions using Nm23 as bait in the yeast two-hybrid system was referred (Gervasi *et al*., 1998). The murine Nm23-M1 protein was used to screen an 11-day mouse embryo cDNA library leading to the isolation of two classes of clones encoding the Nm23-M1 and the Nm23-M2 protein. The reason why no other proteins were isolated from the library is conceivable related to the hexameric structure of eukaryotic NDPKs. X-ray crystallography indicated that the enzymes are trimers of parallel dimers (Morera *et al*., 1995; Webb *et al*., 1995). Murine Nm23-M1 and -M2, as well human Nm23-H1 and -H2 can form *in vivo* and *in vitro* homohexamers or heterohexamers with different ratios of the subunits (Gilles *et al*., 1991; Urano *et al*., 1992). It may be postulated that the affinity of the interaction between the subunits is higher than any other interaction with different proteins. On the other side, the interactions of Nm23 with other proteins might require the assembly of the hexamer. Noteworthy, all the amino acids that differ in the two subunits are located at the outer surface of the hexamers. Thus, the different combinations of the subunits generate different motifs that might be responsible for protein interactions.

Interestingly, some of the *nm23-M1* cDNA clones isolated from the library corresponded to multiple transcripts endowed with different 3′-untraslated regions featuring peculiar structures responsible for mRNA stability and translatability. The expression of the novel transcripts appeared to be modulated during mouse embryo development, reaching the highest levels at the onset of organogenesis, providing a further support to a role played by Nm23-M1 during development. Moreover, the transcripts were also identified in adult mouse in a tissue specific manner. The finding suggests that the rate of expression and tissue-specific distribution of the different transcripts might regulate the expression levels of Nm23-M1 required for embryo development and differentiated tissues maintenance.

Nuclear Orphan Receptors Interact With Nm23 Proteins

A yeast two-hybrid study indicated that members of the ROR/RZR nuclear orphan receptor subfamily interact

with mammalian Nm23 proteins (Paravicini *et al*., 1996). More precisely, rat RZRβ and human RORα were found to strongly interact with Nm23-M2. Control experiments revealed that RORα was also able to interact in vitro with the human Nm23-H1. The Authors speculated on the possibility that the Nm23 proteins could regulate the transcriptional transactivation of either *ROR*α or *RZR*β. Alternatively, they suggested putative synergistic effects of ROR/RZR and NM23-H2 on the N-*myc* gene transcription. The conjecture was supported by the identification in the first intron of the N-*myc* gene a putative response element for ROR/RZR, as well as three putative binding sites for Nm23-H2. Anyway, transient transfection experiments did not confirm both the hypotheses.

Nm23-H1 and -H2 have the ability to both stimulate and repress transcription by binding to positive and negative transcriptional elements and by removing distorted and potentially repressive DNA structures (reviewed in Postel *et al*., 2000). The interaction with ROR/RZR might influence Nm23/NDPK DNA transcriptional properties.

Human Telomeres Interact With Nm23-H2

The yeast two-hybrid system was employed to identify protein that associate with TRF1 (Nosaka *et al*., 1998), a proteins that binds telomeres and controls their length by inhibiting the action of telomerase (van Steensel and de Lange, 1997). The screening led to the isolation from a human HeLa cDNA library of a clone encoding the Nm23-H2 protein. The interaction was also confirmed by *in vitro* protein binding assays. Moreover, further *in vitro* assays indicated that the recombinant Nm23-H2 protein could also bind single-stranded telomeric DNA and the RNA component of telomerase. A telomerase activity associated with Nm23-H2 was not detected. Moreover, whether Nm23-H2 influences TRF1 role as a negative regulator of telomerase, or participates in the assembly of the telomere complex *in vivo* was not demonstrated. Noteworthy, anyway, the zebrafish homologue of the Nm23-H2 protein was demonstrated to *in vitro* bind single-stranded telomeric repeat (Lee and Lee, 2000).

Human PRUNE Interacts With Nm23-H1

The null mutation in the *prune* gene leads to abnormal eye colour in *Drosophila melanogaster* (Timmons and Shearn, 1996). However, the mutation is lethal in the presence of at least one copy of the *killer of prune* (*k-pn*) mutation (Timmons and Shearn, 1997) of the *awd* gene, the *Drosophila* homologue of the *nm23-H1* and *-M1* genes (Rosengard *et al*., 1989). The phenotype suggests a physical interaction between the gene products. The human homologue of *prune* was cloned and the interaction between Nm23-H1 and PRUNE was verified by yeast interaction-mating experiments (Reymond *et al*., 1999). Interestingly, the ability to bind PRUNE was retained by the Nm23-H1 P96S mutant but not by the Nm23-H1 S120G mutant. Proline 96 corresponds to the site of the *k-pn* mutation of the *Drosophila awd* gene (Biggs *et al*., 1988), whereas serine 120 is the site of the mutation found in aggressive human neuroblastoma (Chang *et al*., 1994). The S120G mutation compromises protein stability to denaturation (Chang *et al*., 1996) leading to an incorrect protein folding (Lascu *et al*., 1997). The *P96S* mutation alters protein assembly and subunits interaction (Lascu *et al*., 1992, Karlsson *et al*., 1996). Both mutations affect Nm23-H1 motility inhibition of cancer cells (MacDonald *et al*., 1996) and impair Nm23-H1 ability to promote neural cell differentiation (Lombardi *et al*., 2001). The Authors proposed PRUNE as a negative regulator of Nm23-*H1* and, in a more recent paper (Forus *et al*., 2001), suggested that in tumors such as human breast carcinoma and sarcoma, amplification and overexpression of PRUNE could be a mechanism for inhibiting Nm23-H1 and consequently promoting tumor development and progression.

The Epstein-Barr Virus Protein EBNA-3C Interacts With Nm23-H1

A very interesting report provided the first evidence of an interaction between an oncogenic protein and the metastasis suppressor protein Nm23-H1. The Epstein-Barr virus (EBV) causes infectious mononucleosis and is associated with human cancers as Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease. Among the viral proteins expressed upon infection, EBNA-3C is essential for immortalisation of primary B-lymphocytes. More precisely, the carboxy terminus of EBNA-3C is critical for such functions (Tomkinson *et al*., 1993). This region was used as bait to screen a cDNA library from EBV transformed B-lymphoblastoid cell line (Subramanian *et al*., 2001). The screening revealed a strong interaction with Nm23-H1. The association of EBNA-3C and Nm23-H1 was also confirmed by *in vitro* experiments using a recombinant Nm23-H1 protein and by *in vivo* experiments showing the interaction between endogenous Nm23-H1 and EBNA-3C in human B lymphoblastoid cell lines. Moreover, the Authors demonstrated that EBNA-3C translocates Nm23-H1, usually mainly cytoplasmic, to subnuclear structures. Recently and controversially, EBV was shown to be associated also with malignant breast

carcinoma (Bonnet *et al*., 1999; Brink *et al*., 2000). Noteworthy, EBNA-3C inhibited the ability of Nm23-H1 to suppress the migration of breast carcinoma as well as Burkitt lymphoma cells in an *in vitro* migration assay. EBNA-3C effect on cell migration was observed only in the case of overexpression of *nm23-H1*. It is known that in many tumors high levels of Nm23-H1 correlates with low degree of invasiveness and that the transfection of *nm23-H1* cDNA in cancer cells decreases their metastatic potential (reviewed in De La Rosa *et al*., 1995 and in Lombardi *et al*., 2000). Anyway, it is not clear whether Nm23-H1 translocation to the nucleus by EBNA-3C is merely functional to cell migration or is also the basis for a concerted action of the two proteins in EBV transformed cells.

The Menin Protein Interacts With Nm23-H1

Nonsense, missense, and frame-shifting mutations of the *MEN1* gene, coding the protein referred to as menin, are associated with the familial multiple endocrine neoplasia type 1 (Agarwal *et al*., 1997). Despite the function of *MEN1* is not yet known, it is conceivable that it is a tumor suppressor gene. A yeast two-hybrid analysis using MEN1 as bait to screen a rat foetal forebrain cDNA library revealed an interaction with NDPK β, the rat homologue of human Nm23-H1 (Ohkura *et al*., 2001). Recombinant Nm23-H1 and menin proteins interacted *in vitro*. Anyway, Nm23-H1 did not interact with MEN1 proteins carrying missense mutations, as detected in MEN1 patients. The association was not verified in normal cells and the expression of *nm23-H1* in tumor cells from MEN1 patients was not investigated. Since the interaction was found analysing a library from a foetal tissue in which *MEN1* is highly expressed (Bassett *et al*., 1999), it might be that the association is somehow functional to first stages of development. However, as Nm23-H1 inhibits metastases and promotes differentiation, interplay with the putative tumor suppressor MEN1 might exert a control on tumor growth.

The Integrin Cytoplasmic Domain-Associated Protein 1α (ICAP-1α) Interacts With Nm23-H2

Cell adhesion to extracellular matrix is mediated by integrins clustered in focal complexes linked to the actin cytoskeleton. Integrin cytoplasmic domains recruit many structural and signalling proteins. ICAP-1α interacts specifically with the β1 integrin cytoplasmic tail (Chang *et al*., 1997; 2002) and supports cell migration (Zhang *et al*., 1999). In an attempt to elucidate the molecular basis of ICAP-1α signalling, the protein was used as bait in a yeast two-hybrid system to screen a human placenta library (Fournier *et al*., 2002). The screening besides confirming the interaction with the β1 integrin cytoplasmic domain, revealed a further interaction with Nm23-H2. The interaction was also detected in control *in vitro* and *in vivo* experiments using recombinant and transfected *ICAP-1α* and *nm23-H2*. Confocal fluorescence microscopy indicated a colocalisation of both proteins in lamellipodia and ruffles during early stages of cell spreading on β1 integrin specific substrates. A colocalisation of ICAP-1α and Rac1 was also observed. The Authors concluded that the interaction between ICAP-1α and Nm23-H2 provides a drastically different interpretation of the role of the Nm23 proteins in tumor invasion. Actually, such interpretation seems hazardous since it is widely documented that whereas Nm23-H1 is implicated in the control of cell migration, Nm23-H2 regulates gene expression in agreement with its preferential nuclear localisation. It is not obvious how ICAP-1α translocates Nm23-H2 from the nucleus to cell periphery. Moreover, as referred above in this review, it was shown that Nm23-H1 inhibits Rac1 activation by negatively regulating its exchange factor Tiaml (Otsuki *et al*., 2001). Rac1 induces lamellipodia and membrane ruffles (Sander *et al*., 1999) functional to cell migration, whereas *nm23-H1* overexpression was an inhibitory effect on ruffle and lamellipodium formation (Otsuki *et al*., 2001). In this scenario, it might be conceivable a misinterpretation of the confocal fluorescence microscopy analyses probably due to a cross-reactivity of the anti-Nm23-H2 antibodies with highly homologous Nm23-H1, at least as far as the experimental conditions for cell immunostaining. Thus, the interaction of ICAP-1α and Nm23-H2 detected in yeast, might need to be further analysed.

CONCLUSIONS

The individuation of proteins that interact with Nm23/NDPKs provides new information useful to better delineate their biological functions. Further analysis of Nm23 interactions with human PRUNE, with the putative tumor suppressor MEN1 protein, and with the viral EBNA-3C transforming protein, in different cellular contexts, will lead to the dissection of pathways throughout Nm23-M1 and -H1 inhibit tumor cell invasiveness, interfere with cell proliferation, and promote the differentiated phenotype. On the other side, Nm23 interactions with ROR/RZR transcription factors and with telomeric DNA suggest new inferences about the Nm23-H2 role in gene transcription regulation and DNA structure control.

Moreover, the heterogeneity of the interactions so far identified further indicates that the Nm23 proteins can not be merely regarded as NDPKs, but are multifunctional. In this scenario, it will be noteworthy to investigate whether the interactions require the hexameric structure, known to be essential for the catalytic activity (Lascu *et al*., 1992), or are otherwise mediated by monomeric or dimeric forms of the Nm23 proteins.

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