A new function of Nm23/NDP kinase as a differentiation inhibitory factor, which does not require it's kinase activity

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Abstract **We recently identified a differentiation inhibiting factor (I-factor) in mouse myeloid leukemia M1 cells as a murine homolog of nm23-H2/nucleoside diphosphate kinase (NDPK)-B gene product. We examined the I-factor activities of several authentic nm23/NDPK proteins, i.e recombinant rat NDPK α and β, recombinant mouse nm23-M1 and -M2, and recombinant human nm23-H1 and -H2 containing a mutant nm23-H2^{His} protein lacing NDPK activity. Almost all these nm23/NDPK proteins showed I-factor activity. Moreover, to understand the active domain exhibiting I-factor activity of nm23-H2 protein lacking NDPK activity, we have investigated the I-factor activities of some truncated nm23-H2 proteins. The truncated nm23-H2 protein containing N-terminal peptide 1–60 retained the I-factor activity. These results provide the first evidence for a function of nm23/NDPK as a differentiation inhibiting factor in leukemic cells, that is independent of its NDPK activity and dependent on the presence of N-terminal peptide.**

Key words: Nm23; NDP kinase; Leukemia cell; Differentiation; Inhibition

1. Introduction

Mouse myeloid leukemia M1 cells, established from a spontaneous myeloid leukemia in an SL mouse, can be induced to differentiate along the monocyte/macrophage pathway by various inducers including dexamethasone, 1α,25-dihydroxyvitamin D_3, interleukin 6, and leukemia inhibitory factor/D-factor (LIF/DF) [1,2]. The process of differentiation induced in M1 cells is similar to that of normal myeloid progenitor cells [3].

Activity to inhibit differentiation of M1 cells (I-factor activity) was detected in a cell lysate and conditioned medium of the differentiation-resistant M1 cells, but not in those of the parental cells [4,5]. Moreover, the production of I-factor activity in these resistant M1 cells was well associated with their development of resistance to differentiation inducers [6]. Recently, we purified one of the I-factors from conditioned medium of differentiation-resistant M1 cells. The purified I-factor had a relative molecular mass of approximately 16,000–17,000 Da, and its amino acid sequence determined from cyanogen bromide fragments was identical with that of nm23 proteins, which are involved in tumor metastasis regulation and contain nucleoside diphosphate kinase (NDPK) enzyme activity (EC2.74.6) [7]. These results suggested that the nm23/NDPK protein might be the I-factor that suppresses differentiation of leukemic cells.

nm23 was first identified as a gene expressed in lower amounts in highly metastatic rodent tumors than in poorly metastatic tumor cells [8]. Homologs of nm23 have been identified in development of *Drosophila* as *awd* [9] and of *Dictyostelium* as *gip* 17 [10]. The deduced amino acid sequences of the products of these nm23 genes were found to share high homology with NDPK in a variety of species. The nm23 proteins, as well as their evolutionary homologs, have been demonstrated to have NDPK activity, although it has not been established that the enzyme activity mediates the biological effect of nm23 [11–13]. In humans, NDPK A and B are identical to two isotypes of human nm23 homologs, named nm23-H1 and -H2, respectively [12,14]. Two isotypes of nm23 homologs nm23-M1 and -M2, have also been found in mice [15]. Recently, another type of NDPK (NDPK-β) in addition to NDPK-α, was identified in rats [16]. It is, therefore, very likely that mammalian nm23/NDPKs have two isotypes with high mutual homology. Our previous results on amino acid sequence analysis showed that the purified I-factor is probably nm23-M2 [7,15], but it is unknown whether the I-factor activity requires the NDPK activity of nm23 protein.

In the present work, we examined (i) whether nm23/NDPK proteins have I-factor activity, and (ii) whether the NDPK activity is essential for the differentiation inhibitory activity of I-factor.

2. Materials and methods

2.1. Cells and cell culture

M1 cells were cultured in Eagle's minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan), supplemented with double the usual concentrations of amino acids and vitamins and 10% (v/v) heat-inactivated (56°C for 30 min) calf serum at 37°C under 5% CO_2 in air.

2.2. Assay of properties of differentiated cells

Phagocytic activity was assayed using polystyrene latex particles, as reported previously [5]. Lysozyme activity in the cells was determined by the lysoplate method described previously [17]. Locomotive activity was assayed by the colonies with or without cell migration, as reported previously [1,5].

2.3. Assay of I-factor activity

M1 cells (2×10^5/ml) were cultured for 2 days in the presence of dexamethasone (2×10^{-8}M) with or without a test preparation. The ability of the preparation to inhibit the induction by dexamethasone of

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Abbreviations: I-factor, a differentiation inhibitory factor of mouse myeloid leukemia M1 cells; NDPK, nucleoside diphosphate kinase; GST, glutathione *S*-transferase; LIF/DF, leukemia inhibitory factor/D-factor: PAGE, polyacrylamide gel electrophoresis.

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phagocytic activity of cells was measured, and the I-factor activity was calculated as the percentage inhibition as reported previously [4,5].

2.4. nm23/NDPK proteins

Rat recombinant NDPK proteins (α and β) were produced in *E. coli* and purified and characterized as reported previously [18]. Murine and human recombinant nm23 proteins (nm23-M1, -M2, -H1, and -H2) were produced as fusion proteins with 26 kDa glutathione *S*-transferase (GST) in *E. coli* and purified [15,19]. Using site-directed mutagenesis of cDNA encoding nm23-H2, we created the mutant nm23-H2^{His} protein, where His^{118} was replaced by Cys because His^{118} was the substrate for phosphoenzyme formation by ATP [20]. The mutant nm23-H2^{His} protein was produced as fusion protein with GST in *E. coli*. To create the truncated nm23-H2 proteins, plasmids were constructed to express the N-terminal peptide1-60 (nm23-H2-AX-CX), N-terminal peptide1-108 (nm23-H2-AX-DX), and C-terminal 61–152 (nm23-H2-EX-BX) of nm23-H2 proteins. Primers used for PCR of 5′-end (1–180)] region, 5′-end (1–324) region, and 3′-end (181–456) region of nm23-H2 gene coding regions were as follows: Sense primers: H2-AX, 5′-GGGGATCCATGGCCAACCTGGAGCG-3′; H2-EX, 5′-GGGGATCCTTCCCTGGGCTGGTGAA-3′ (each primer contained a *Bam*HI site). Antisense primers: H2-CX, 5′-GGGAATTCAGAATGGTCGGTCTTTCA-3′; H2-DX, 5′-GGGAATTCAGAAGTCCCCACGAATGG-3′; H2-BX, 5′-GGGAATTCATTCATAGACCCAGTCAT-3′ (each primer contained a *Eco*RI site). All the nm23 GST fusion proteins were purified to homogeneity on SDS-PAGE by the procedure described previously ([15,19] Fig. 1A). Their NDPK activities were assayed by the phosphoenzyme formation of NDPK. Neither the mutant nm23^{His} protein nor the truncated nm23-H2 proteins contained NDPK activity (Fig. 1B). All the proteins were dialyzed against phosphate buffered saline before the assay of the I-factor activity.

3. Results

3.1. I-factor activity of recombinant nm23/NDPK proteins

The I-factor activities of purified recombinant nm23/NDPK proteins were examined. All the nm23/NDPK proteins used (human nm23-H1 and -H2, mouse nm23-M1 and -M2, and rat NDPK-α and -β) inhibited the dexamethasone-induced differentiation of M1 cells (Fig. 2). Although nm23-H1, -H2, -M1, and -M2 were fusion proteins with GST, GST alone did not show any I-factor activity (Fig. 2A). Their I-factor activities were observed at very low concentrations of nm23/NDPK proteins, and at higher concentrations their I-factor activities decreased. At their effective concentrations, nm23/NDPK proteins had no effect on proliferation of M1 cells. When differentiation was induced by dexamethasone, the presence of excess differentiation inducer counteracted the I-factor activity of nm23-M2 (data not shown). There were no marked differences in the I-factor activities of the isoforms of nm23/NDPK proteins (Fig. 2), although in some experiments nm23-M2 and -H2 had higher I-factor activities than nm23-M1 and-H1. Next, we tested the inhibitory activity of nm23-M2 on dexamethasone-induced locomotive activity and lysozyme activity which were functional and biochemical markers of monocytic differentiation of M1 cells. The nm23-M2 protein also inhibited both locomotive and lysozyme activities (Table 1). The results indicate that all the purified recombinant nm23/NDPK proteins tested inhibited the differentiation of M1 cells, and showed no species specificity.

3.2. I-factor activity does not require NDPK activity

To understand the active domain of nm23/NDPK protein for I-factor activity, we examined the I-factor activities of some mutant nm23/NDPK proteins. One is the mutant nm23-H2^{His}, which has Cys^{118} instead of His^{118} and does not have NDPK activity (Fig. 1B). This mutant cannot form a phosphoenzyme by ATP because His^{118}, which is the substrate for phosphoenzyme formation by ATP, is replaced by a Cys residue [20]. The mutant nm23-H2^{His} protein showed I-factor activity like wild type nm23-H2 protein (Fig. 3). These results suggest that NDPK activity is not required for the I-factor activity of nm23/NDPK protein. Thus nm23/NDPK protein may have a new function, independent of its kinase activity.

The other mutants used were truncated nm23-H2 proteins, which were N-terminal (1–60) peptide (nm23-H2-AX-CX), N-terminal (1–108) peptide (nm23-H2-AX-DX), and C-terminal (61–152) peptide (nm23-H2-EX-BX) of nm23-H2 protein. These truncated nm23-H2 proteins were also produced in *E. coli* in fusion form with GST and purified (Fig. 1). They were laking the NDPK activity (Fig. 1B). The N-terminal peptides (nm23-H2-AX-CX and nm23-H2-AX-DX) showed higher I-factor activity than the C-terminal peptide (nm23-H2-EX-BX) (Fig. 4). The N-terminal peptides inhibited not only the induction by dexamethasone of phagocytic activity but also that of locomotive and lysozyme activities of M1 cells (data not shown). These results suggest that at least the presence of the

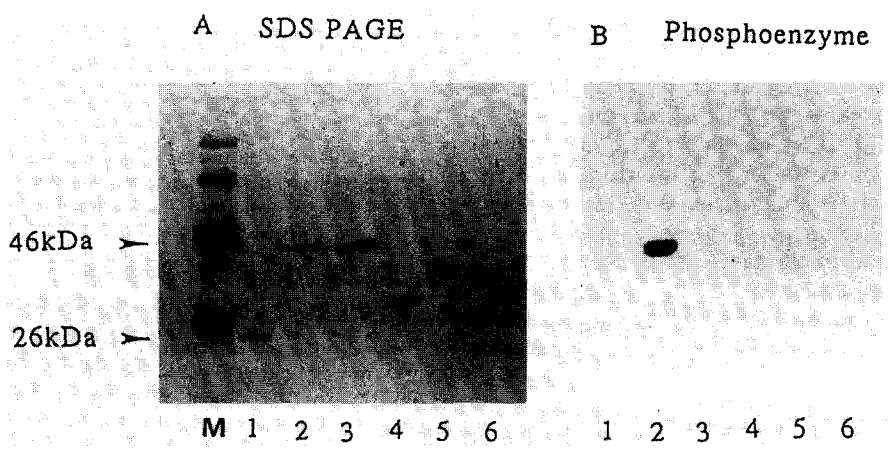

Fig. 1. (A) SDS-PAGE analysis of purified nm23 H2 GST fusion proteins. The gel was stained with Coomassie blue dye. (B) NDPK activity of the purified nm23-H2 GST fusion proteins detected by the phosphoenzyme intermediate by autoradiography. The proteins (0.5 μg) used were GST (lane 1), nm23-H2 (lane 2), nm23-H2^{His} (lane 3), AX-CX (lane 4), AX-DX (lane 5) and EX-BX (lane 6). M, marker proteins.

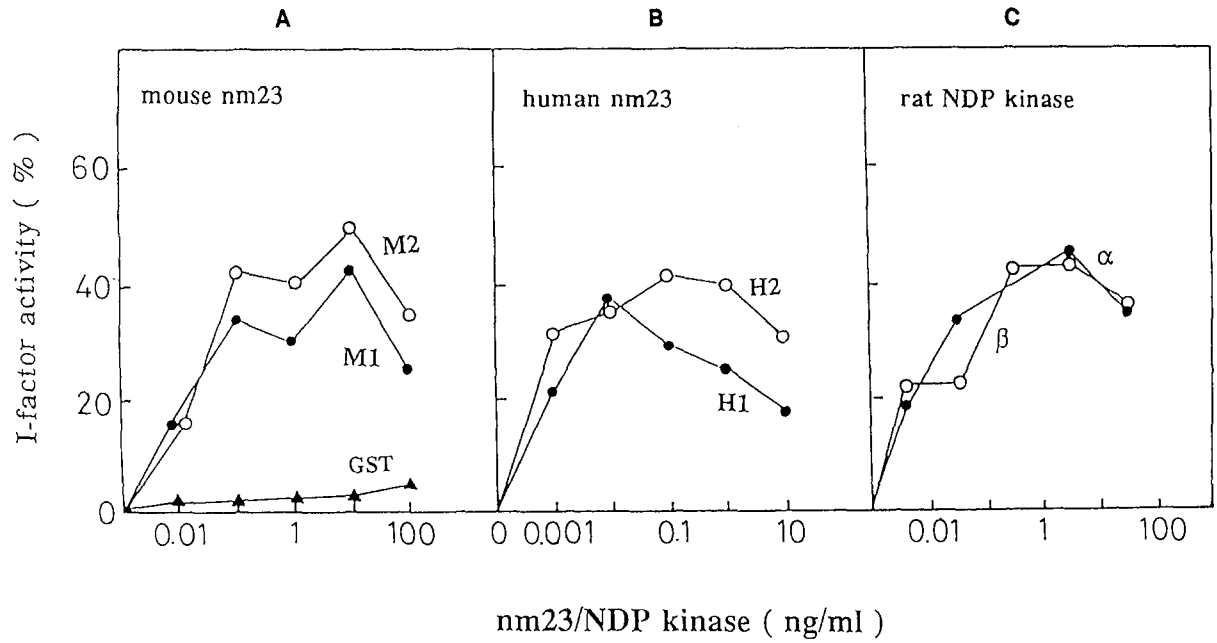

Fig. 2. I-factor activities of various recombinant nm23/NDPK proteins. The phagocytic activities of control cells in the presence of dexamethasone were (A) 52.8 ± 1.9% (n = 4 S.D.), (B) 53.8 ± 3,9% (n = 4, S.D.), and (C) 58.9 ± 3.6 (n = 3, S.D.).

N-terminal region of nm23-H2 protein is required for the I-factor activity.

4. Discussion

nm23 genes are implicated in tumor metastasis control through as yet unknown mechanisms. Depending on the cell type, metastatic spread has been correlated both with reduced nm23 expression [21–23], and with overexpression of nm23 [24]. nm23 genes are also involved in controlling normal cellular functions, including cell proliferation [25], differentiation [7,26], motility [27], and development, as indicated by high degree of homology between nm23 genes and the *Drosophila awd* gene vital to normal fruit fly development [9]. All nm23/awd proteins contain NDPK enzyme activity [10–13], catalysing the synthesis of non-adenine-containing nucleoside triphosphates from nucleoside diphosphates. Additionally nm23/NDPK proteins have been described to interact with GTP-binding proteins [10,28], bind chromoglycate dye [29], autophospharylate serine residue [30], and bind to DNA and stimulate transcription [31]. Of these functions of nm23/NDPK proteins, the antimetastatic potential of nm23 and the gene regulatory function (PuF) have been recently showed the independence with NDPK activity [30,32]. Moreover, MacDonald et al. presented thet a serine phosphorylation of nm23 correlated with suppression of tumor metastatic potential [30]. In the present study, we examined the correlation between the NDPK activity and I-factor activity of nm23 proteins. Our results showed that the I-factor

Table 1
Effect of nm23/NDP kinase on various markers of differentiation of M1 cells

Marker of differentiation	nm23-M2	Differentiation inducer	
		None	Dex[c]
Phagocytic activity (%)	–	5.8 ± 1.8	53.8 ± 4.9
	+[a]	4.8 ± 0.8	20.0 ± 1.4
Locomotive activity (%)	–	0	47.5 ± 15.3
	+[b]	0	10.4 ± 5.3
Lysozyme activity (Units/mg protein)	–	0	1.48
	+[b]	0	0.87

[a]0.1 ng/ml of nm23-M2; [b]1 ng/ml of nm23-M2; [c]2×10^{-8} M dexamethasone; Values are means ± S.D. (n = 4).

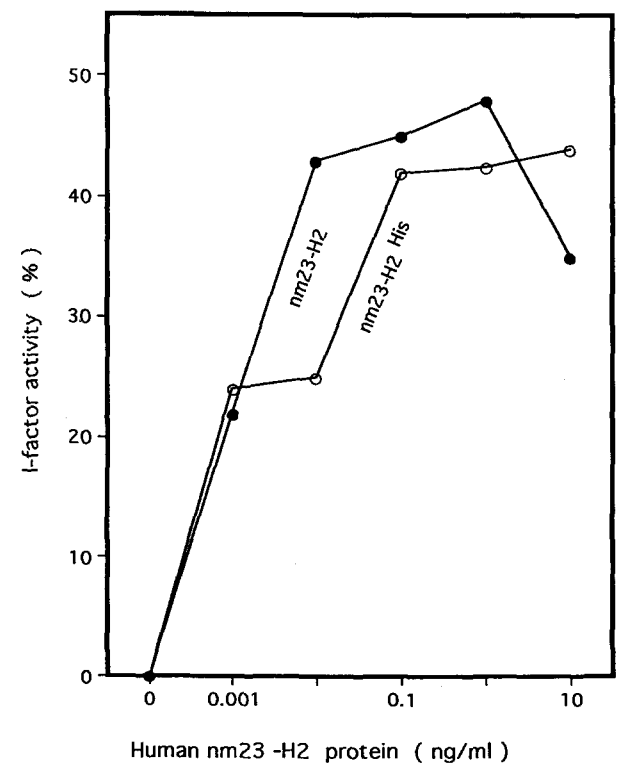

Fig. 3. I-factor activity of mutant nm23-H2 protein. The phagocytic activity of control cells in the presence of dexamethasone was 57.3 ± 9.7% (n = 7, S.D).

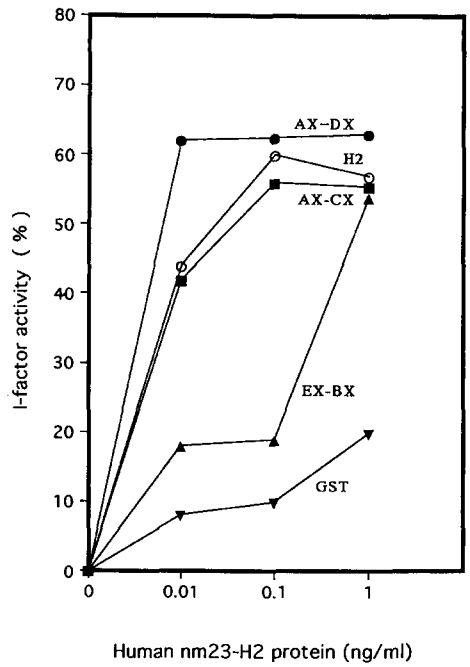

Fig. 4. I-factor activity of truncated nm23-H2 proteins. The phagocytic activity of control cells in the presence of dexamethasone was 56.5 ± 4.3% (n = 4, S.D.).

activity is not mediated by its kinase activity (Figs. 3 and 4). Namely, nm23/NDPK proteins may have another function that is independent of their NDPK activity.

We are now trying to determine the active domain of nm23 protein required for its I-factor activity. All previously identified nm23/NDPKs, except that in bacteria, share the tripeptide, Arg-Gly^{106}-Asp, which is the RGD consensus sequence for the recognition of integrin family members. The I-factor activity in M1 cells has been reported to be mainly localized in the membrane fraction [4]. Recently, the presence of nm23/NDPK protein on the cell surface of some line cells has been reported [33], although the molecular mechanism of the surface expression of nm23/NDPK protein is unknown. An intriguing possibility is that nm23/NDPK functions as a ligand for adhesive molecules of the integrin family. However, the truncated nm23-H2 proteins, lacking C-terminal peptide containing the RGD consensus sequence, exhibited I-factor activity (Fig. 4). On our preliminary experiment, the mutant nm23-H2^{RGD} protein, which has Ser^{106} instead of Gly^{106}, also showed I-factor activity like wild type nm23-H2 protein (unpublished data). These results suggest that the I-factor actitivity is not mediated by the RGD consensus sequence of nm23 protein. The I-factor activity of nm23-H2 protein required the presence of N-terminal peptide (Fig. 4), containing leucine zipper motif, a serine phosphorylation site (Ser^{44}), and a mutation site (Leu^{48}) reported in aggressive childhood neuroblastomas. The mechanism by which I-factor inhibits induction of differentiation of M1 cells is unknown. nm23/NDPK are mainly localized in the nucleus and cytosol with little in the plasma membrane [33]. We purified the I-factor from the culture medium of M1 cells, although nm23/NDPK has no signal peptide for its secretion [5,7]. Recently, we found nm23 protein with NDPK activity in the culture supernatants of a variety of cell lines (T. Urano, K. Furukawa, and H. Shiku, manuscript in preparation), although the molecular mechanism of its secretion is unknown.

On the basis of their nuclear localization and potential leucine zipper motif, nm23-H2 and nm23-H1 were proposed to function as a transcription factors [14,21]. Postel et al. presented evidence that nm23-H2 is a transcription factor (PuF) and that one of its targets is the c-*myc* gene [31]. Recently, they have reported that the NDPK activity of nm23-H2/PuF protein is not required for its function as a transcription factor [32]. It is interesting to investigate whether the c-*myc* transcription factor activity of nm23/H2 protein is required for its I-factor activity. Although no clear correlation has yet been established between overexpression of c-*myc* and tumor metastasis, the inverse relation between c-*myc* expression and cell differentiation is well documented. In M1 cells, the expression of c-*myc*, which is high during proliferation, is suppressed following induction of terminal differentiation and growth arrest [34]. Liebermann and Hoffman–Lieberman found that deregulated and continued expression of c-*myc* in M1 cells inhibited terminal differentiation of the cells [35]. Our identification of I-factor in M1 cells as the murine homolog of nm23-H2 suggests that the possible interaction between c-*myc* and nm23 may occur during block of differentiation of leukemic cells.

In conclusion, the present data provide the first evidence for nm23 function as a differentiation inhibitory factor in leukemic cells independent of its NDPK activity and dependent of the presence of its N-terminal region of nm23/I-factor protein.

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