Transactivation potential of the C-terminus of human Nm23-H1

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Abstract The transactivation potential of Nm23-H1, a homolog of *c-myc* transcription factor Nm23-H2/PuF was assessed in yeast as a fusion protein with the DNA binding domains (DBDs) of GAL4 and LexA. The C-terminal half of Nm23-H1 exhibited strong transactivation of the reporter genes, *LacZ* and *Leu2* carrying GAL4 and LexA upstream activating sequences (UASs), whereas the full-length Nm23-H1 and its N-terminal did not. Similar results were also obtained with Nm23-H2/PuF transactivating the reporter genes only by the C-terminus fused to GAL4 and LexA DBDs. Hence, our results suggested a possible regulatory role of the N-termini of Nm23 isotypes upon transactivation.

Key words: Nm23-H1; Nm23-H2; PuF; NDP kinase; Transactivation

1. Introduction

Nm23-H1 was first identified as a tumor metastasis suppressor [1]. The inverse relationship between the metastatic potential and the level of Nm23-H1 expression was well established in various cancers [2–4]. Nm23-H1 and its homolog proteins are well conserved from *Escherichia coli* to human. These proteins have NDP kinase activities catalyzing ATP-dependent synthesis of nucleoside triphosphates from their corresponding nucleoside diphosphates [5]. Nm23 homologs in various higher eukaryotes have been proposed to retain multifunctional properties. Involvements of Nm23 homologs in various cellular processes, such as stimulating transcription [6,7], cell differentiation and proliferation [8,9], and apoptosis [10] have been described. In addition, a serine/threonine specific protein phosphotransferase activity [11,12] and a histidine protein kinase activity [13] were also shown to be detected.

In human, four Nm23 isotypes, Nm23-H1, Nm23-H2, DR-Nm23 and Nm23-H4 are known to date [10,14–16]. Among them, Nm23-H1 and Nm23-H2 shared highest amino acid homology [15] and coexist as heterohexamers [17–19]. However, these two isotypes have been considered to participate in different cellular processes. Nm23-H1 has demonstrated a metastasis suppressor function whereas Nm23-H2 has not. Nm23-H2 was proved to be identical to PuF, a transcription factor of *c-myc* proto-oncogene [6], but Nm23-H1 failed to bind to the *c-myc* promoter [20]. Recombinant Nm23-H2/PuF made from *E. coli* was able to bind to the nuclease-hypersensitive element within the *c-myc* promoter [7] and a single-stranded polypyrimidine-rich DNA and RNA [20] in vitro.

Considering high homology to Nm23-H2/PuF, it was of much interest whether Nm23-H1 might function as a transcription factor. However, a direct test of DNA binding capacity of Nm23-H1 was not possible since no target sequence, if any, was identified yet. Thus, we assessed a transactivation potential of Nm23-H1 with creating fusion proteins to the known DNA binding domains with the aid of yeast genetic system. As well, in order to minimize the artifacts as a fusion protein, two independent assay systems, *GAL4*- [21,22] and *LexA*-based [23] systems, were used.

2. Materials and methods

2.1. Materials, media, strains, and plasmids

All chemicals used for yeast transformation and β-galactosidase assays were purchased from Sigma (St. Louis, MO). Restriction enzymes, ligase, and Klenow fragment were obtained from Promega (Madison, WI). Synthetic dropout minimal (SD) medium with various supplements was used for yeast culture, maintenance, and selection of transformants [24]. Yeast strains, Y187 (*Matα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, URA3::GAL1*UAS-*GAL1*TATA-*lacZ*), and EGY48 (*Mata, ura3-52, his3, trp1, leu2::pLeu2-LexAop6*/pSH18-34 (*LexAop-lacZ*)) were obtained from Clontech Laboratories, Inc. (Palo Alto, CA). As yeast-*E. coli* shuttle vectors, pGBT9 carrying yeast GAL4 DBD [25] and pLexA containing *E. coli* LexA DBD sequences [26] were purchased from Clontech Laboratories, Inc. as parts of the MatchMaker two-hybrid assay kits. Plasmids pET3C-*nm23-H1* and pET3C-*nm23-H2* [27,28] carrying the coding sequences of *nm23-H1* and *nm23-H2*, respectively, were kindly provided by Dr. P.S. Steeg (NIH, USA).

2.2. Construction of vectors expressing the Nm23-H1 and the Nm23-H2 full-size or truncated proteins fused to GAL4 and LexA DNA binding domains

The *Nde*I-*Bam*HI fragments from the pET3C-*nm23-H1* and the pET3C-*nm23-H2* corresponding to the Nm23-H1 and the Nm23-H2 coding regions, respectively, were inserted into *Sma*I and *Bam*HI double digested pGBT9. The *Nde*I sites of the inserts were filled-in with Klenow enzyme to generate in-frame fusion to the GAL4 DBD. The resulting plasmids were named pT9H1 and pT9H2, respectively. The correct reading frames for expressing Nm23-H1 and Nm23-H2 in pT9H1 and pT9H2 were verified by sequencing. For the construction of N-terminal halves (1 to 76 amino acids of total 152 amino acids) of Nm23-H1 and Nm23-H2, pT9H1 and pT9H2 were cut with *Nco*I/*Bam*HI enzymes, then the larger fragment was religated after filling-in the protruding both ends with Klenow enzyme. Similarly, in order to get the C-terminal halves (76 to 152 amino acids), each of the larger *Nco*I/*Eco*RI fragments of pT9H1 and pT9H2 was rejoined after Klenow fill-in, creating in-frame fusion with the GAL4 DBD coding region. These plasmids were called pT9H1N (producing the N-terminal half of Nm23-H1 fused to GAL4 DBD), pT9H2N (N-terminal half of Nm23-H2), pT9H1C (C-terminal half of Nm23-H1), and pT9H2C (C-terminal half of Nm23-H2).

For the *LexA* based assay alternative to the *Gal4* system, plasmids pLexH1 and pLexH2 were constructed by inserting the 0.5 kb *Eco*RI-

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Abbreviations: DBD, DNA binding domain; AD, activation domain; UAS, upstream activating sequence; SD medium, synthetic dropout minimal medium; ONPG, *o*-nitrophenyl β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; CAT, chloramphenicol acetyltransferase

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*Sal*I fragments containing the Nm23-H1 and the Nm23-H2 coding sequences from pT9H1 and pT9H2 into *Eco*RI/*Sal*I double digested pLexA vector, respectively. The N-terminal and C-terminal halves of Nm23-H1 and Nm23-H2 fused in-frame to the LexA DBD were also constructed by Klenow fill-in/ligation of the larger *Nco*I/*Sal*I or *Nco*I/*Eco*RI fragments of pLexH1 and pLexH2. These were named pLexH1N (N-terminal half of Nm23-H1 fused to GAL4 DBD), pLexH1C (C-terminal half of Nm23-H1), pLexH2N (N-terminal half of Nm23-H2), and pLexH2C (C-terminal half of Nm23-H2). The correct reading frames were confirmed by sequencing.

2.3. Yeast transformation and β-galactosidase assay

The lithium acetate method for preparing yeast competent cells [29] was adopted to introduce the various constructs into yeast. Transformants were selected on SD medium lacking proper supplements as indicated in each case below.

The β-galactosidase enzyme assays of colony-lift filters [21] and of liquid cultures using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate [30] were carried out according to the instructions from Clontech Laboratories, Inc. In the plate β-galactosidase assay for a blue/white detection directly on SD medium lacking uracil and histidine for pLexA derived constructs, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) was added at a concentration of 40 mg/l to melted SD medium (48°C). Blue colors on the colony-lift filters and on the plates containing X-gal were judged after 3 days at 30°C.

3. Results

3.1. Transactivation of the lacZ reporter gene by the C-terminal half of Nm23-H1 in the Gal4 based assay system

Transactivation of the *lacZ* reporter gene with GAL4 UAS was tested by introducing fusion proteins of Nm23-H1 with GAL4 DBD in yeast cells. Various plasmids expressing either full-size (namely, pT9H1), N-terminal half (pT9H1N) or C-terminal half (pT9H1C) of the Nm23-H1 were transformed into yeast host Y187, and then the transformants selected on SD medium depleted tryptophan were assayed for the β-galactosidase enzyme activity. For the comparison, similar constructs, namely pT9H2, pT9H2N and pT9H2C made for Nm23-H2 were also transformed. Such fusion proteins can specifically bind to the upstream region of the *lacZ* reporter gene.

In the colony-lift β-galactosidase filter assay for a blue/white color detection, the transformants of pT9H1 and pT9H1N exhibited white color. On the contrary, blue color was developed in a short time in the transformants of pT9H1C producing truncated Nm23-H1 proteins having the C-terminal half (data not shown because of color prints). Comparable results were obtained in the transformants expressing the Nm23-H2/Gal4 fusion proteins, showing blue colors also only in the transformants of pT9H2C producing the C-terminal half of Nm23-H2. For the quantitative comparison, the liquid β-galactosidase assay using ONPG as a substrate was applied to those transformants. As shown in Fig. 1, β-galactosidase units in the transformant of pT9H1C were significantly increased ($P < 0.01$) more than 10-fold compared to those detected in transformants of pT9H1 and pT9H1N showing the negative control level (see also Table 1). Similar results were also obtained with Nm23-H2 (Fig. 1). Even though the C-terminal half of Nm23-H2 transactivated the reporter gene less strongly than that of Nm23-H1, yet higher than those found in the full-size and the N-terminal half of Nm23-H2 proteins ($P < 0.01$). Blue signals for the evidence on transactivation of the reporter gene by the full-size proteins of Nm23-H1 and Nm23-H2 were absent or too weak to detect.

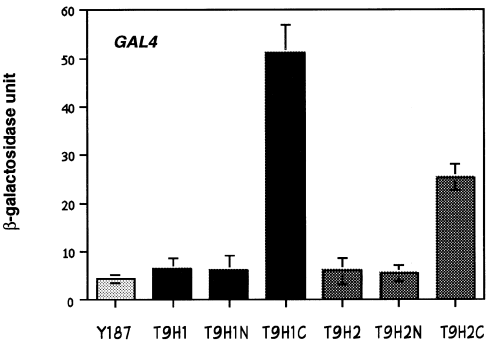

Fig. 1. Differential transactivation potentials of the truncated versus the full-size Nm23 isotypes in *GAL4* system. The liquid β-galactosidase assay was carried out in the transformants of yeast strain Y187 expressing the fusion proteins of GAL4 DBD with Nm23-H1 full size (from pT9H1), Nm23-H1 N-terminus (pT9H1N), Nm23-H1 C-terminus (pT9H1C), Nm23-H2 full size (pT9H2), Nm23-H2 N-terminus (pT9H2N), and Nm23-H2 C-terminus (pT9H2C). One unit of β-galactosidase activity was defined as the amount of enzyme required to hydrolyze 1 μmol of ONPG to *o*-nitrophenol and D-galactose per minute. The average values with standard errors of β-galactosidase units were indicated based on the five sets of independent experiments. Each set assaying β-galactosidase activities of the six kinds of transformants plus a negative control (Y187) was carried out at the same time. Duplicate measurements for each sample were performed.

3.2. Transactivation of the lacZ reporter gene in the LexA based assay system by the Nm23-H1 C-terminus

To confirm the observed results and exclude the possible artifacts in the *Gal4* based assay, fusion proteins of Nm23-H1 as well as Nm23-H2 to LexA DBD were expressed and tested for transactivation of the *lacZ* reporter gene having LexA UAS in yeast. The transformants of pLexH1 producing the full-length Nm23-H1 with LexA DBD, pLexH1N (N-terminal halves), and pLexH1C (C-terminal halves) were selected on SD medium without uracil and histidine. Comparable constructs of pLexH2, pLexH2N, and pLexH2C for Nm23-H2 were also transformed into EGY48. Then, the β-galactosidase enzyme assay for color developments was carried out on SD plates containing X-gal. Similarly shown in the *Gal4* based assay, only the transformants of pLexH1C as well as pLexH2C expressing the C-terminal half of each protein turned blue, while colonies of pLexH1 and pLexH2 producing the full-length proteins remained white (data not shown).

Table 1
Fold differences of β-galactosidase unit in the various transformants compared to that of host cells, Y187 for the *GAL4* and EGY48 for the *LexA* based systems

	Host cell	H1	H1N	H1C	H2	H2N	H2C
GAL4 system	1	1.5	1.4	12	1.4	1.3	6
LexA system	1	2.1	5.4	523	3.2	7.4	116

Such differences were clear and extreme in the liquid β-galactosidase assay (Fig. 2). More than 100–250-fold increase for β-galactosidase enzyme units was demonstrated in the transformants of pLexH1C compared to those detected in the transformants of pLexH1N and pLexH1 (Table 1). The transactivation of the *LacZ* reporter was driven more strongly (more than 7-fold increase) by the C-terminus of Nm23-H1 than by the B42, a weak activator domain derived from bacteria fused to LexA [31], as a positive control for transactivation (Fig. 2). Again, activation of the *lacZ* gene by the full length of Nm23-H1 and Nm23-H2 fused to LexA DBD was not able to be detected. In addition, much higher transactivation of the *lacZ* were exhibited in the transformants of pLexH1C compared to that of pLexH2C ($P < 0.01$).

3.3. Transactivation of the Leu2 reporter gene by the C-terminal half of Nm23-H1 in the LexA system

The yeast strain, EGY48 has dual reporter genes, the *LacZ* within the pSH18-34 plasmid and the yeast *LEU2* with *LexA* operators integrated into yeast chromosome. Without transcriptional stimulation of the *Leu2* gene, the transformants with pLexA derived plasmids could not grow on SD lacking leucine. Fig. 3 showed the transformants of pLexH1, pLexH1N, and pLexH1C grown on SD medium lacking uracil and histidine for positive growth (Fig. 3A) and SD medium depleting uracil, histidine, and leucine to check transactivation of the *Leu2* reporter (Fig. 3B). Full growth of pLexH1C transformants on the medium without leucine (Fig. 3B; bottom-left) indicated strong transactivation of the *Leu2* reporter gene by the Nm23-H1 C-terminal half. Growth of the transformants of pLexH1 was hardly detected (see Fig. 3B; top-left), implying no transactivation of *Leu2* gene by the full-size Nm23-H1 proteins. In the case of transformants of pLexH1N expressing N-terminal halves of Nm23-H1 fused to LexA DBD, colonies grew very slowly and eventually can be seen as spots after 4–6 days on SD medium lacking leucine (Fig. 3B; middle-left). Similar results for Nm23-H2/PuF's transactivation of the *Leu2* reporter (see Fig. 3; right-half of the plates) were demonstrated in the transformants of pLexH2 (top-right), pLexH2N (middle-right), and pLexH2C (bottom-right) producing the full-length, N-terminal, and C-terminal halves of Nm23-H2, respectively.

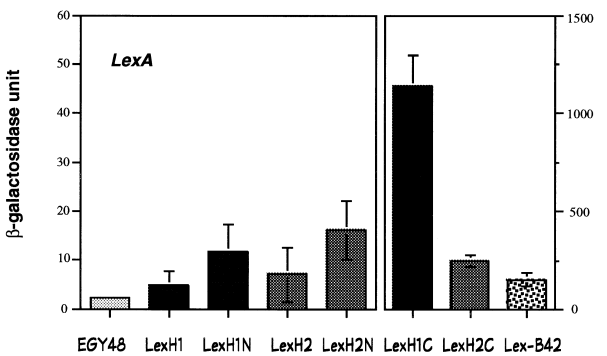

Fig. 2. Differential transactivation potentials of Nm23 domains. The liquid β-galactosidase assay was performed in the transformants of yeast strain EGY48 producing fusion proteins of LexA DBD with Nm23-H1 full size (from pLexH1), Nm23-H1 N-terminus (pLexH1N), Nm23-H1 C-terminus (pLexH1C), Nm23-H2 full size (pLexH2), Nm23-H2 N-terminus (pLexH2N), and Nm23-H2 C-terminus (pLexH2C). The average values with standard errors of β-galactosidase units were indicated based on the five sets of independent experiments. Each set of experiments including a negative control (EGY48) was carried out simultaneously and each sample was measured in duplicate. As a positive control (pLex-B42), the transformants expressing the known activation domain of B42 derived from bacteria fused to LexA were used. Definition of one β-galactosidase unit as described in Fig. 1. (Note different scale for LexH1C, LexH2C, and Lex-B42.)

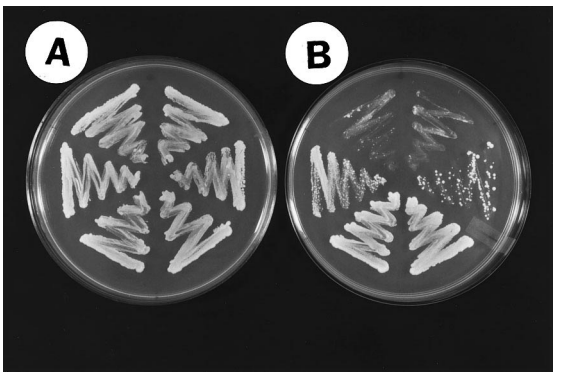

Fig. 3. Growth dependency on transactivation of the *Leu2* reporter gene. The transformants of EGY48 with pLexH1 (top-left in both A and B plates; producing the full-length Nm23-H1 fused to LexA DBD), pLexH1N (middle-left; N-terminus of Nm23-H1), pLexH1C (bottom-left; C-terminus of Nm23-H1), pLexH2 (top-right; full size of Nm23-H2/PuF), pLexH2N (middle-right, N-terminus of Nm23-H2), and pLexH2C (bottom-right, C-terminus of Nm23-H2) were streaked on SD medium without uracil and histidine (plate A) and lacking uracil, histidine, and leucine (plate B). The plates were incubated at 30°C for 5 days at the same conditions.

4. Discussion

The fusion proteins of Nm23 with the well characterized DBDs such as the yeast GAL4 and the *E. coli* LexA were expressed in suitable hosts carrying the reporter genes of *LacZ* and *Leu2* with GAL4 and LexA UASs, in order to test whether the human Nm23-H1 possesses a transactivation domain. From the assays using two alternative *Gal4* and *LexA* based systems, clearly we obtained the consistent results: (1) the truncated protein having only the C-terminal half of Nm23-H1 was able to transactivate the reporter genes, and (2) the full-length Nm23-H1 failed to transactivate the reporters or transactivated too weak to detect. Results obtained here suggested the possibility of Nm23-H1 as a putative transcription factor comparable to Nm23-H2/PuF, even though both of the full-length Nm23 isotypes were not able to stimulate transcription of the reporters. Apparently, deletion of the N-terminal halves unmasked the potential transactivation capability of Nm23 proteins.

A negative effect upon transactivation by the full length of both proteins was not due to the lack of expression of both proteins and the failure of nuclear translocation. In the yeast two-hybrid assay, cotransformants of the Nm23-H1/GAL4 DBD fusion vector (pT9H1) and the Nm23-H2/GAL4 activation domain (AD) fusion vector showed blue color on X-gal plates (data not shown). Similarly, cotransformation of the Nm23-H2/GAL4 DBD fusion vector (pT9H2) and the Nm23-H1/GAL4 AD fusion vector gave the same result. These positive signals not only demonstrated the protein interactions between Nm23-H1 and Nm23-H2 but also provided the evidence of the proper expressions and nuclear translocations of both Nm23 isotypes.

Nm23-H2 monomer consists of four stranded antiparallel β-sheets whose surfaces are partially covered by six α helices [32]. The truncated C-terminal half used in this study contained the regions of α3, α4, α5, and β4. Moreover, the C-terminal half carried the active site of NDP kinase as well as Kpn loop [9]. In order to test whether the structures of the truncated C-terminal halves of Nm23 proteins could be maintained as the original topology, NDP kinase activities and autophosphorylations in truncated proteins have been measured. It turned out that the C-terminal halves of both Nm23 isotypes still demonstrated the NDP kinase activities and also retained the autophosphorylation capabilities (will be published elsewhere). This indicated that the structure of the C-terminal halves was relatively well maintained even after deletion of the N-terminal halves.

Recently, Michelotti et al. reported that the full-length Nm23-H1 and the Nm23-H2/PuF fused to the GAL4 DBD failed to transactivate the bacterial chloramphenicol acetyltransferase (CAT) reporter gene using a transient transfection assay [33]. Our data agreed well with the results of Michelotti et al. However, these results disagreed to the earlier finding of Berberich and Postel where the *myc*-CAT expression could be driven by Nm23-H2 in vivo [7]. The reason for such a discrepancy is still not clear. However, both Michelotti's and Berberich's papers suggested a possible involvement of a cofactor(s) in influencing transcription by Nm23 proteins in vivo. Since Nm23-H1 was known to form heteromers with Nm23-H2 [17,19], the possibility of Nm23-H2 as a putative cofactor influencing the transactivation capability of Nm23-H1 was tested. However, our unpublished results excluded such a possibility. In order to find other putative cofactors, searching proteins interacting with Nm23 isotypes are currently underway using the yeast two-hybrid screening system. The identities of these proteins might elucidate the mechanism of Nm23 in transcriptional machinery.

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