Tumour metastasis suppressor, nm23- β , inhibits gelatinase A transcription by interference with transactivator Y-box protein-1 (YB-1)

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Gelatinase A transcriptional regulation is the consequence of combinatorial interactions with key promoter and enhancer elements identified within this gene. A potent 40 bp enhancer response element, RE-1, located in the near 5' flanking regions of the rat and human gelatinase A genes drives high-level expression in glomerular mesangial cells (MCs). Southwestern-blot analysis of MC nuclear extracts revealed specific interactions of RE-1 with at least four proteins, of which three have been identified as p53, activator protein 2 and the single-stranded DNA-binding factor Y-box protein-1 (YB-1). In the present study, we report the identification of a fourth 17 kDa RE-1-binding protein as the rat homologue (nm23- β) of the human nm23-H1 metastasis suppressor gene. Recombinant nm23- β protein bound only the single-stranded forms of the RE-1 sequence. Mutagenesis revealed direct interaction of nm23- β with a repeat sequence, 5'-GGGTTT-3', shown previously to specifically interact with YB-1 [Mertens, Harendza, Pollock and Lovett (1997) *J. Biol. Chem.* **272**, 22905–22912], and recombinant nm23- β protein competed for single-stranded YB-1 binding. Transient transfection of MC

with an nm23- β expression plasmid within the context of a RE-1/simian virus 40 promoter/luciferase reporter yielded a concentration-dependent repression (80–90%) of luciferase activity in MC and Rat1 fibroblasts. A similar pattern of nm23- β repression was demonstrated within the context of the RE-1/homologous gelatinase A promoter. Co-transfection of nm23- β blocked YB-1-mediated activation of transcription and expression of gelatinase A. Nm23- β may be an important physiological regulator of gelatinase A transcription that acts by competitive interference with the single-stranded transactivator YB-1. Gelatinase A is a key mediator of tumour metastasis, suggesting that competitive suppression of transcription by nm23- β (or the human nm23-H1) may be a component of the reduced metastatic capabilities of cells expressing high levels of this protein.

Key words: MMP-2, matrix metalloproteinase, nucleoside diphosphate kinase.

INTRODUCTION

The matrix metalloproteinases (MMPs)-2 and -9 (also denoted gelatinase A and B, respectively) are essential mediators of key physiological and pathological processes, including morphogenesis, neo-angiogenesis, tumour metastasis and arthritis [1,2]. Due to the central roles of the gelatinases in these complex biological processes, stringent transcriptional and post-translational regulatory controls exist to regulate synthesis in spatially and temporally defined patterns. Although gelatinase A and B have very similar substrate specificities against a broad spectrum of extracellular matrix molecules, the transcriptional regulatory patterns of these two genes are distinctive. In common with many members of the MMP gene family, the promoter region of the gelatinase B gene includes a TATA box and activator protein (AP)1, nuclear factor κ B (NF- κ B) and polyoma enhancer activator 3 ('PEA3') sites [3,4]. In sharp contrast, the 5' promoter regions of the rodent and human gelatinase A genes lack TATA or CAAT boxes, as well as conserved AP1 or NF κ B consensus sequences [5,6]. Extensive deletion analyses of the rat gelatinase A 5' flanking region have identified previously a potent tissue-specific 40 bp enhancer response element, RE-1, located between –1322 and –1282 bp relative to the translational start site [5]. A highly homologous sequence, denoted r2, is present within a similar region of the human gelatinase A gene and also functions as a potent enhancer element [7,8]. Southwestern blots of glomerular

mesangial cell (MC) nuclear extracts, using the RE-1 as a probe, have demonstrated specific interactions with several critical transcription factors, two of which have been identified as AP2 and Y-box protein-1 (YB-1) [9,10]. YB-1 is a highly conserved DNA-binding factor with a primary affinity for single-stranded, as opposed to double-stranded, DNA [9]. These observations prompted a further examination of MC nuclear extracts using the single-stranded components of RE-1 as probes and revealed specific interactions with a 17 kDa nuclear protein. We report the identification of this single-stranded RE-1-binding protein as the rat homologue (nm23- β) of the human metastasis suppressor nm23-H1. Nm23- β specifically binds to a repeat sequence, 5'-GGGTTT-3', within the RE-1 sequence and suppresses gelatinase A transcription by direct competition with transactivator YB-1 binding.

MATERIALS AND METHODS

Cells and culture conditions

The isolation and characterization of rat glomerular MCs has been described in detail previously [11]. MCs were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 μ g/ml streptomycin and 100 units/ml penicillin. Rat1 fibroblasts and HEK-293 cells

Abbreviations used: AP, activator protein; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; GST, glutathione S-transferase; MC, mesangial cell; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1-MMP; NF- κ B, nuclear factor κ B; ORF, open reading frame; RE-1, response element-1; ss1, sense; ss2, antisense; SV40, simian virus 40; $t_{1/2}$, half-life; YB-1, Y-box protein-1.

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were obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.) and maintained in the same growth medium.

Plasmids

The upstream 1686 bp relative to the translational start site of the rat gelatinase A gene, which includes RE-1 and the homologous promoter [5], were subcloned into the promoterless luciferase expression vector pGL2-Basic (Promega, Madison, WI, U.S.A.) and designated as pT4-Luc1686. The 40 bp RE-1 sequence, $-^{1322}$ AGCCTGCTGGGCAAGTCTGAACCTGTCA-GAAACCCACTAG $-^{1282}$, was subcloned into the vector pGL2-Promoter (Promega), which includes the heterologous simian virus 40 (SV40) promoter, and was designated as pT4-Luc1322BP. The nm23- α isoform was cloned by reverse transcription-PCR of MC mRNA using the primers 5'-GCACCATGGCCA-ACCTCGAGCGTACCTTCATT-3' [sense (ss1)] and 5'-CTCC-CCGTCTACTACATACACCCAGTCATGG-3' [antisense (ss2)], and cloned into pSG5 (Stratagene, La Jolla, CA, U.S.A.). A YB-1 expression vector (pSG5-YB-1) was a gift from Dr Jenny P.-Y. Ting (Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, U.S.A.) and consisted of the complete YB-1 open reading frame (ORF) cloned into the expression vector pSG5 (Stratagene). The β -galactosidase expression vector pCMV- β -gal (ClonTech, Palo Alto, CA, U.S.A.) was used as a transfection standardization control.

Nuclear extracts

MCs were grown to 90% confluence in 150-mm culture dishes, washed twice with ice-cold PBS without calcium and magnesium, and scraped into 10 ml of ice-cold PBS. Nuclear extracts were prepared as described by Dignam et al. [12]. Proteins were extracted in a buffer containing 20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT) and 0.2 mM PMSF and dialysed overnight in a similar buffer containing 1 M NaCl instead of KCl. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. Nuclear extracts were stored at -80°C prior to electrophoretic mobility-shift assays (EMSAs) and Southwestern-blot analysis.

Southwestern-blot analysis

Oligonucleotides used corresponded to the ss1 and ss2 strands of the 40 bp RE-1 sequence detailed above. Double-stranded oligonucleotide probe was generated using standard methodology. Nuclear extracts (30 μg of protein) were electrophoresed on SDS/10% (w/v) polyacrylamide gels and transferred by electroblotting on to nitrocellulose membranes. The membranes were blocked in a buffer containing 25 mM Hepes (pH 8.0), 10% (v/v) glycerol, 50 mM NaCl, 1 mM EDTA and 2.5% (w/v) dried milk powder for 12 h at 4°C , washed for 5 min in TNE-50 buffer [10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA and 1 mM DTT] and probed for 4 h at 30°C in TNE-50 containing 10 $\mu\text{g}/\text{ml}$ poly(dI-dC) and radiolabelled oligonucleotide (10^6 c.p.m./ml). Synthetic oligonucleotides were end-labelled with polynucleotide kinase and [γ - ^{32}P]ATP for single-stranded-binding assays. Double-stranded probes were prepared by digesting pT4-Luc1322BP with *Asp*718 or *Bgl*II. The resultant overhanging 5'-ends were filled in with [α - ^{32}P]ATP by means of the Klenow fragment of DNA polymerase I, followed by digestion with *Bgl*II and *Asp*718 respectively. Single- and double-stranded probes were purified on acrylamide gels and eluted.

EMSAs

For DNA-binding studies, MC nuclear extracts (10 μg) or indicated concentrations of recombinant YB-1 and nm23- β proteins were mixed with radiolabelled oligonucleotides (5×10^4 c.p.m.) and incubated at 22°C for 30 min in $5 \times$ binding buffer A [20 mM Hepes (pH 7.9), 20% (v/v) glycerol, 0.1 M NaCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT] containing 300 $\mu\text{g}/\text{ml}$ acetylated BSA and 500 ng of poly(dI-dC). The samples were electrophoresed on 4% polyacrylamide/7.5% glycerol gels in a buffer containing $1 \times$ Tris/borate/EDTA [100 mM Tris/90 mM borate and 1 mM EDTA (pH 8.0)], followed by autoradiography. For competition studies, unlabelled oligonucleotides were added to the nuclear extracts in the concentrations indicated at the time of addition of the radiolabelled probes. Dissociation rates of nm23- β binding to the respective ss1 and ss2 components of the RE-1 were determined by incubating the radiolabelled oligonucleotides as detailed above with 2.5 ng of recombinant nm23- β protein per reaction for 30 min at 22°C . Thereafter, a 500-fold molar excess of unlabelled competitor oligonucleotide was added. At the time points indicated in the Figure legends, samples were immediately subjected to electrophoresis and the amounts of shifted labelled ss1 and ss2 oligonucleotide were quantified by densitometry.

For antibody supershifts, nuclear extracts were incubated overnight at 4°C with 4 μg of murine monoclonal anti-(rat nm23- β) antibody (Transduction Laboratories, San Diego, CA, U.S.A.) or control murine IgG, prior to addition of labelled oligonucleotides and electrophoresis. Two additional controls for the specificity of the antibody supershift experiments were performed. In the first, the murine monoclonal anti-(nm23- β) antibody was coupled to AminoLink agarose (Pierce) using the manufacturer's instructions. A portion (50 μl) of the coupled nm23- β antibody beads was incubated with 150 μl of MC nuclear extract for 2 h at 4°C . The beads were then pelleted and the supernates used for EMSA in the presence or absence of antibody as detailed above. In the second control, recombinant nm23- β protein was coupled to the AminoLink agarose beads and 50 μl of beads were incubated with the monoclonal anti-(nm23- β) antibody. The absorbed antibody solution was then used in the EMSA as detailed above.

Expression cloning

A rat MC λ gt11 cDNA expression library was prepared using standard methodology and cloned into the λ ZipLox phage expression vector (SuperScript λ System; Gibco BRL, Gaithersburg, MD, U.S.A.). The library was screened as described by Singh et al. [13] with a radiolabelled single-stranded oligonucleotide corresponding to the non-coding sequence of RE-1. Nitrocellulose membranes (Hybond-C; Amersham Biosciences, Piscataway, NJ, U.S.A.) were washed in TNE-50 for 10 min at 24°C and blocked for 12 h at 4°C with blocking buffer A. Hybridization with the radiolabelled oligonucleotide and washing were as described above. Corresponding positive signals in duplicate lifts were purified to homogeneity by secondary and tertiary screens. Cloned λ phage cDNA was isolated by transformation of *Escherichia coli* DH10B(ZIP), yielding excised cDNA in plasmid vector pZL1. cDNA inserts were sequenced with the TaqTrack sequencing system (Promega).

Protein expression and purification

The cDNAs encoding the ORFs of YB-1 and nm23- β were cloned into the bacterial expression vector pRSET (Invitrogen, Carlsbad,

CA, U.S.A.). For expression in *E. coli*, bacteria were induced with isopropyl β -D-thiogalactoside for 2 h at 37 °C, followed by lysis in B-PER solution (Pierce) and serial freeze thawing. The hexahistidine fusion proteins were purified to homogeneity on Ni²⁺-affinity columns according to the manufacturer's instructions (Invitrogen). Purity of the recombinant proteins was ascertained by analytical SDS/PAGE and Western blotting.

Figure 1 Southwestern-blot analysis of MC nuclear extracts

Double-stranded (DS) RE-1 probe hybridized to three bands of molecular mass 86, 62, and 52 kDa. The 62 kDa band corresponded to phosphorylated YB-1, and the 52 kDa band to both AP2 and YB-1. The single-stranded RE-1 probes (ss1 and ss2) also hybridized to a 17 kDa protein not detected with the double-stranded probe.

Transient transfections

Transient transfection of MCs, Rat1 fibroblasts and HEK-293 cells were performed with Lipofectin (Life Technologies, Gaithersburg, MD, U.S.A.) as described previously [9]. For each quadruplicate transfection, 1 μ g of the corresponding luciferase construct (pGL2-Basic, pT4-Luc1686 or pT4-Luc1322BP) and 1 μ g of normalizing CMV- β gal vector were mixed in 100 μ l of Optimem (Gibco BRL) and 20 μ g of Lipofectin diluted into 100 μ l of Optimem. For co-transfection studies, the indicated concentrations of the pSG5-nm23- β or pSG5-nm23- α expression plasmids were added to the Optimem solution containing the DNA. Both solutions were incubated at 24 °C for 45 min and then combined. After incubation for a further 15 min, 800 μ l of Optimem was added. The amount of total DNA was equalized in each reaction by inclusion of the empty plasmid pSG5. The plasmid-containing Optimem mixtures were added to washed cultures of cells and incubated overnight, after which the Optimem was removed and replaced with normal growth medium. Cells were grown for a further 24 h, washed once with ice-cold PBS and extracted with 400 μ l of Triton lysis buffer [1% (v/v) Triton X-100, 1 mM DTT, 25 mM glycylglycine (pH 7.8) and 15 mM MgSO₄] on ice for 30 min. Luciferase activity was measured with 100 μ l of the lysate as described by Brasier et al. [14], and β -galactosidase was determined using the Luminescent β -gal Detection Kit (ClonTech). All transfections were performed in quadruplicate and were repeated at least three times. Transfection results were averaged and are expressed as means $\pm$ S.D.

Quantitative zymography

To determine the effects of nm23- β and nm23- α expression on the rates of gelatinase A protein synthesis, quantitative gelatin zymography was performed as described previously [8] on serum-free medium (containing 0.1% BSA) harvested 48 h after transfection with 1 μ g of control pSG5 plasmid, 1 μ g of pSG5-nm23- β or 1 μ g of pSG5-nm23- α . Zymography of the co-transfections with YB-1 and nm23- β was performed in a similar fashion. A portion (25 μ l) was suspended in non-reducing sample buffer and

Figure 2 EMSA with recombinant nm23- β protein and the radiolabelled ss1 and ss2 strands of RE-1

Incubation of radiolabelled oligonucleotides with recombinant nm23- β protein (5 ng) was performed as described in the Materials and methods section. The electrophoretic mobilities of both strands are reduced by recombinant nm23- β protein. Unlabelled (cold) oligonucleotide competition studies indicated a significantly higher affinity of nm23- β for the ss2 component of RE-1, whereas the unlabelled scrambled sequences did not compete for binding.

analysed by densitometric scanning of developed gelatin–SDS/PAGE substrate gels. The densitometric activity of 10 pg of recombinant gelatinase A (Oncogene Research Products, San Diego, CA, U.S.A.) was assigned a value of 1. Data are expressed as means ± S.D. of quadruplicate determinations.

RESULTS

Southwestern-blot analyses of glomerular MC nuclear extracts revealed distinctive hybridization patterns when probed with either double- or single-stranded forms of the RE-1 sequence (Figure 1).

Figure 3 Comparison of the relative affinity of recombinant nm23-β for ss1 versus ss2 oligonucleotides

The dissociation rates of radiolabelled ss1 and ss2 oligonucleotides from a fixed concentration (2.5 ng) of recombinant nm23-β protein were determined as described in the Materials and methods section. The $t_{1/2}$ for binding of nm23-β to the ss1 and ss2 strands was 1.9 and 4.5 min respectively.

The double-stranded RE-1 probe delineated three specific bands with apparent molecular masses of 86, 62 and 52 kDa. As reported previously [9], the 52 kDa band consists of both AP2 and YB-1, whereas the 62 kDa band corresponds to the phosphorylated form of YB-1. The 86 kDa band has been recently identified as signal transduction and activator of transcription 1 (‘STAT1’; D. H. Lovett and P. R. Mertens, unpublished work). The single-stranded components of RE-1 demonstrate significant binding to 52 kDa YB-1 and very prominent binding to a 17 kDa nuclear protein not seen with the double-stranded probe.

To identify the RE-1 single-stranded-binding nuclear protein, we used an expression screening approach with a rat MC λgt11 cDNA library and a radiolabelled single-stranded RE-1 oligonucleotide (ss2 strand). After screening 1.5×10^6 primary recombinants, six hybridizing plaques were identified by duplicate lift methods. These plaques were carried through two additional hybridization cycles to homogeneity. Sequence analysis of all six clones revealed an identical sequence encoding the 737 bp ORF of rat nm23-β, the homologue of human nm23-H1 [15].

EMSAs with recombinant nm23-β confirmed the single-stranded-binding activity observed with the Southwestern blots (Figure 2). Binding of the radiolabelled ss1 strand of the RE-1 sequence was specifically competed by the addition of 100–250-fold concentrations of unlabelled oligonucleotide. The binding affinity of recombinant nm23-β for the ss2 strand of the RE-1 sequence was significantly greater. Oligonucleotides composed of the scrambled sequences of ss1 and ss2 did not compete for nm23-β binding, consistent with a sequence requirement for binding (see below). To quantitatively determine the relative binding affinity of nm23-β protein to the respective ss1 and ss2 oligonucleotides, a formal dissociation rate analysis was performed. The results of these studies are shown in Figure 3. The half-life ($t_{1/2}$) for nm23-β binding to the ss1 strand was 1.9 min, whereas

Figure 4 EMSA of MC nuclear extract with single-stranded RE-1 oligonucleotides

MC nuclear extract (MCNE) reduced the electrophoretic mobilities of the labelled RE-1 single-stranded oligonucleotides. Supershifts (arrow) were obtained with both ss1 and ss2 using the monoclonal anti-nm23-β antibody. To confirm the specificity of supershifted bands for nm23-β, additional controls (middle panel) included the use of monoclonal antibody-pre-treated MCNE (Ab-Rx) and monoclonal antibody pre-incubated with recombinant nm23-β protein (nm23-β-Rx). The results for only ss1 are shown, but the results for ss2 are identical.

Figure 5 Co-transfection of nm23- β reduces the luciferase reporter activity of the RE-1 sequence in conjunction with the intrinsic gelatinase A promoter

The nm23- β expression plasmid (pSG5-nm23- β) was co-transfected with the luciferase (luc) reporter construct pT4-Luc 1686 into the indicated cell types as described in the Materials and methods section. The plasmid pT4-Luc 1686 contains the RE-1 sequence. The luciferase activity of the control pGL2-Basic vector was assigned a value of 1. Results are expressed as relative luciferase activities and are means $\pm$ S.D. of quadruplicate determinations.

Figure 6 Co-transfection of nm23- β reduces the luciferase reporter activity of the RE-1 sequence in conjunction with a heterologous SV40 promoter

The nm23- β expression plasmid (pSG5-nm23- β) was co-transfected with the luciferase (luc) reporter construct pT4-Luc 1322BP into the indicated cell types as described in the Materials and methods section. The plasmid pT4-Luc 1322BP contains the RE-1 sequence in conjunction with a heterologous SV40 promoter. The luciferase activity from the control pGL2-Promoter (pGL2-Prom) plasmid was assigned a value of 1. Results are means $\pm$ S.D. of quadruplicate determinations.

the $t_{1/2}$ for the ss2 strand was 4.5 min, consistent with approx. a 2.5-fold greater affinity of the nm23- β protein for the ss2 strand.

To extend these observations to interactions within the context of MC nuclear extracts, EMSA was performed using a specific monoclonal anti-(nm23- β) antibody (Figure 4). Although inclusion of pre-immune sera did not affect the electrophoretic mobility of major shifted complexes, inclusion of the specific

monoclonal anti-(nm23- β) antibody yielded clear supershifts for both ss1 and ss2 probes, consistent with a specific interaction of the nm23- β protein with MC nuclear extracts. To confirm the specificity of the antibody-mediated supershifts for the nm23- β protein, two complementary additional controls were performed. In the first, nuclear extracts were pre-incubated with immobilized monoclonal anti-(nm23- β) antibody. EMSA of these nm23- β -depleted nuclear extracts no longer demonstrated an

Figure 7 EMSA of recombinant nm23-β with mutated ss1 and ss2 within a 6 bp sequence at position -1293 to -1287

5'-AAACCC-3' in ss1 was mutated to 5'-AAAGGG-3' (ss1mut) and the complementary sequence in ss2, 5'-GGGTTT-3', was mutated to 5'-GGGAAA-3' (ss2mut). Using equal amounts of probe and 2.5 ng of nm23-β, the mutations in ss1 and ss2 abolished nm23-β binding.

antibody-dependent supershift (Figure 4, middle panel). The second control consisted of pre-absorption of the monoclonal anti-(nm23-β) antibody with recombinant nm23-β protein. As shown in Figure 4 (middle panel), pre-absorption of the antibody resulted in loss of the supershifted complex.

To assess the biological significance of nm23-β interaction with RE-1 in terms of transcriptional activity, transient transfection experiments were performed using luciferase reporter constructs and an nm23-β expression plasmid. Cells chosen for these transient transfection experiments included rat glomerular MCs, Rat1 fibroblasts and HEK-293 cells. Figure 5 shows the results of experiments in which the luciferase reporter construct contained the RE-1 sequence with the intrinsic gelatinase A proximal promoter (pT4-Luc1686). Co-transfection with the nm23-β expression plasmid resulted in very significant concentration-dependent inhibition of luciferase reporter activity in rat MCs and Rat1 fibroblasts, whereas luciferase activity in HEK-293 cells was not affected. Transcriptional repression was specific to the β isoform, as transfection with the α isoform in MCs did not affect luciferase activity (Figure 5).

Co-transfection of the nm23-β expression plasmid with a reporter construct consisting of the RE-1 sequence with a heterologous SV40 promoter (pT4-Luc1322BP) also resulted in a concentration-dependent inhibition of luciferase activity (Figure 6). Reporter activity in MCs and Rat1 fibroblasts was reduced by approx. 10-fold at the highest concentration of nm23-β expression plasmid, whereas reporter activity in transfected HEK-293 cells was suppressed between 2–3-fold. As with the studies shown in Figure 5, co-transfection with 4 μg of the nm23-α expression plasmid did not affect RE-1-dependent luciferase activity. Taken together, these experiments suggest that nm23-β suppresses gelatinase A transcription by interaction with the RE-1 sequence.

Previous studies [9] concerning gelatinase A transcriptional regulation have defined a key role for the single strand-specific binding factor, YB-1, as a positive transactivator. DNA-protection-footprinting analysis defined two distinct regions of

Figure 8 Mapping of nm23-β-interaction sites within the ss1 and ss2 oligonucleotides

The nm23-β-interaction sites within ss1 were determined by measuring the degree of competition of radiolabelled wild-type ss1 (upper panel) and ss2 (lower panel) oligonucleotides with unlabelled site-specific mutated oligonucleotides. The location of the mutated bp in the ss1 and ss2 sequences is underlined. Unlabelled competition by the respective wild-type ss1 (wt ss1) and ss2 (wt ss2) oligonucleotides was assigned a value of 100%. The degree of competition by 500-fold excess of unlabelled mutated oligonucleotides was determined by densitometry. Results are means ± S.D. of quadruplicate determinations.

YB-1 interaction with the RE-1 sequence. These consisted of interaction with the relatively conserved Y-box sequence, as well as an extended sequence on the ss2 strand composed of 3'-GAACAGTCTTTGGGTG-5' (bp -1279 to -1264), where the underlined bases were specifically footprinted by recombinant YB-1 [9]. Inspection of the ss2 strand revealed the sequence 5'-GGGTTT-3', which is the inverse of the telomeric DNA repeat sequence 5'-TTAGGG-3' recently shown to bind nm23-H2 (the human homologue of rat nm23-α) [16]. These observations suggested that the transcriptional inhibitory activity of nm23-β could be the consequence of interference with YB-1 binding.

To address this issue, three discrete experimental approaches were taken. In the first, EMSA was performed with recombinant nm23-β protein using control RE-1 oligonucleotides and a series of RE-1 oligonucleotides in which the sequences corresponding to the telomeric DNA repeat sequence were mutated. In the

Figure 9 Recombinant YB-1 and nm23- β proteins compete for binding to RE-1

Radiolabelled ss1 and ss2 oligonucleotides were incubated with a fixed concentration of recombinant YB-1 protein (2 ng) and with increasing concentrations of recombinant nm23- β as indicated. Binding of YB-1 on both the ss1 and ss2 strands was competitively displaced by recombinant nm23- β protein.

Figure 10 Co-transfection of nm23- β inhibits YB1-mediated transactivation of RE-1 in conjunction with the heterologous SV40 promoter

The YB-1 expression plasmid (pSG5-YB-1) and increasing amounts of a nm23- β expression plasmid (pSG5-nm23- β) were co-transfected with the luciferase (luc) reporter construct pT4-Luc 1322BP into MCs. The plasmid pT4-Luc 1322BP contains the RE-1 sequence in conjunction with a heterologous SV40 promoter. The luciferase activity from the control pGL2-Promoter (pGL2-Prom) plasmid was assigned a value of 1. Results are expressed as the means $\pm$ S.D. of quadruplicate determinations.

ss1 strand 5'-AGCCTGCTGGGCAAGTCTGAACTTGTCAG-AAACCCACTAG₋₁₂₈₂-3', the sequence underlined (AAACCC) was mutated to AAAGGG. In the ss2 strand 5'-CTAG-TGGGTTTCTGACAAGTTCAGACTTGCCAGCAGGCT-3', the underlined sequence GGGTTT was mutated to GGGA-AA. The results are shown in Figure 7 and demonstrate that mutation of the 5'-AAACCC-3' and 5'-GGGTTT-3' regions of ss1 and ss2 strands respectively, resulted in a loss of nm23- β protein binding activity.

To more specifically define the binding sites of nm23- β within these sequences, competition EMSAs were performed using radiolabelled wild-type ss1 and ss2 oligonucleotides incubated with 500-fold excess of unlabelled mutated oligonucleotide. Each ss1 mutant contained a single bp mutation in the sequence 5'-AAACCC-3', with mutation of each base in the sequence to guanine. Within the 5'-GGGTTT-3' ss2 sequence, each base was sequentially mutated to adenine. The competition activities of the unlabelled wild-type ss1 and ss2 oligonucleotides were

assigned activities of 100%, and the competition activities of the respective unlabelled mutated oligonucleotides were determined by quantitative densitometry and expressed as the percentage of competition activity as compared with the wild-type control. These results are shown in Figure 8 and demonstrate that within the ss1 sequence, 5'-AAACCC-3', the bases in bold are the dominant interactors with nm23- β , and within the ss2 sequence, 5'-GGGTTT-3', the bases in bold are required.

A second set of gel-shift studies was performed to directly assess the ability of recombinant nm23- β protein to compete for YB-1 binding. These experiments are shown in Figure 9. Using a fixed concentration of recombinant YB-1 protein (2 ng), gel-shift studies were performed with the inclusion of increasing concentrations of nm23- β protein. Concentrations of nm23- β protein as low as 1 ng partially inhibited YB-1 binding, and complete competition was observed on the ss1 strand with 2.5 ng of nm23- β protein. Complete competition was nearly obtained with 5 ng of nm23- β protein on the ss2 strand (Figure 9) and

complete competition was obtained with 7.5 ng of nm23- β (results not shown). The stoichiometry of these competition binding assays suggests that YB-1 and nm23- β have relatively similar binding affinities (within one log range) for the RE-1 sequence.

The third form of analysis of YB-1/nm23 β competition assessed the consequences of co-transfection of the nm23- β cDNA on YB-1-mediated transactivation by using the pT4-Luc1322BP/luciferase reporter construct. As shown in Figure 10, co-transfection with 0.5 μ g of YB-1 expression plasmid resulted in an expected 2.5-fold increase in luciferase activity.

Figure 11 Transfection of MCs with an nm23- β expression plasmid inhibits gelatinase A secretion

MCs were transfected with 0.5 μ g of pSG5 plasmid (control), increasing amounts of the nm23- β expression plasmid (pSG5-nm23- β) or 0.5 μ g of the nm23- α expression plasmid (pSG5-nm23- α). After 48 h, the conditioned media were evaluated for gelatinase A activity by SDS/PAGE quantitative gelatinase zymography. The densitometric value obtained with 10 pg of recombinant gelatinase A was assigned a value of 1. Data are given as means $\pm$ S.D. of quadruplicate determinations.

Co-transfection with increasing concentrations of the nm23- β expression plasmid yielded significant decreases in luciferase reporter activity, with activity suppressed by over 3-fold following co-transfection with 0.5 μ g of nm23- β expression plasmid. Co-transfection with the nm23- α expression plasmid did not affect YB-1-mediated transactivation. Taken together, this series of experiments demonstrates the direct competition of nm23- β protein for YB-1 binding, with resultant inhibition of YB-1-mediated transactivation.

To determine whether the observed nm23- β -mediated decrease in gelatinase A reporter construct activity correlated with changes in actual gelatinase A synthetic rates, quantitative zymography was used to measure gelatinase A activity in glomerular MC culture supernatants. MCs were transfected with 0.5 μ g of control pSG5 vector or increasing concentrations of the pSG5-nm23- β expression plasmid or 0.5 μ g of the pSG5-nm23- α expression plasmid. After 48 h, the conditioned media were harvested and subjected to quantitative gelatin-SDS/PAGE zymography. As shown in Figure 11, nm23- β transfection reduced MC gelatinase A secretion in a concentration-dependent fashion, whereas co-transfection with the nm23- α expression plasmid had no effect. The suppressor effects of nm23- β transfection on gelatinase A synthesis was overcome by co-transfection with increasing amounts of the YB-1 expression plasmid (Figure 12). Taken together, the results in Figures 11 and 12 demonstrate the competitive nature of the YB-1-nm23- β interactions with RE-1, which determines the ultimate levels of gelatinase A protein synthesis.

DISCUSSION

The present study has identified rat nm23- β , the homologue of the human nm23-H1, as a single-stranded DNA-specific competitive repressor of YB-1-mediated gelatinase A transactivation. The specific interaction of recombinant nm23- β protein with defined regions within the gelatinase A RE-1 sequence was confirmed by EMSA and specific mutagenesis experiments. Most significantly, nm23- β expression in cultured glomerular MCs resulted in a highly significant isoform-specific concentration-dependent reduction in gelatinase A protein synthesis that could

Figure 12 Transfection of MCs with increasing amounts of a YB-1 expression plasmid antagonizes inhibition of gelatinase A secretion by the nm23- β expression plasmid

MCs were transfected with either 1 μ g of a control pSG5 plasmid or 1 μ g of the nm23- β expression plasmid pSG5-nm23- β alone or with increasing amounts of the YB-1 expression plasmid pSG5-YB-1. After 48 h, the conditioned media were evaluated for gelatinase A activity by SDS/PAGE quantitative gelatinase zymography. The gelatinase A activity in the control pSG5 supernatants was assigned a value of 100%. Results are means $\pm$ S.D. of quadruplicate determinations.

be antagonized by YB-1, thereby linking the transcriptional phenomena to a relevant cell biological outcome.

The nm23- β protein was originally identified as a novel gene associated with reduced tumour metastatic potential [17]. Subsequent studies used transfection of the murine nm23- β cDNA into highly metastatic murine melanoma cells and demonstrated reduced tumour formation and distal metastasis formation in syngeneic and nu/nu mice [18]. These initial transfection studies were confirmed using tumour cells of other origins, including B16F10 melanoma cells and human MDA-MB-435 breast carcinoma cells [19,20]. A substantial literature (reviewed in [21]) has attempted to correlate, with varying degrees of success, nm23- β levels in multiple human cancers with clinical outcome and development of distal metastases.

Nm23 consists of a highly conserved gene family of which four discrete members have been identified in humans to date. These include nm23-H1, nm23-H2, DR-nm23 and nm23-H4 [22]. A common feature of these proteins is the ability to act as a nucleoside diphosphate ('NDP') kinase, which catalyses the transfer of phosphates between nucleoside and deoxynucleoside tri- and di-phosphates. These enzymes are ubiquitously expressed and can form complex homo- and heteromeric structures, although the functional significance of these multienzyme complexes remains unresolved [23–25]. Although nm23-H1 and nm23-H2 are highly related at the sequence level (88% overall similarity), discrete functional differences have been ascribed to these proteins. For example, the metastasis suppression activity originally reported in the murine melanoma model is limited to the H1 isoform, whereas the H2 isoform has not been demonstrated to suppress metastasis [26]. Nm23-H2 is identical with the transcription factor PuF, which drives c-myc proto-oncogene transcription, a function for which nucleoside diphosphate kinase activity is not required [27,28]. Nm23-H2 does not function as a conventional transcription factor, at least when assessed in a yeast two-hybrid reporter system [29]. Nm23-H2 does manifest a high binding affinity for single-stranded pyrimidine-rich DNA sequences, which has been offered as an explanation for the binding of this factor to the PuF site in the c-myc promoter [30]. Indeed, this pattern of binding by nm23-H2 has led to the suggestion of a more generalized structural role in the facilitation of certain DNA conformations conducive to transcriptional processes [31].

Nm23-H1 (or the homologous rat nm23- β) has not been identified previously as a specific DNA-binding protein. In the present study, we have demonstrated the preference of nm23- β for the single-stranded DNA components of RE-1 and have determined through mutagenesis analysis that specific nucleotides are required for the maintenance of nm23- β -DNA interaction. Specific mutations of the sequence 5'-AAACCC-3' on the ss1 strand of RE-1 and the sequence 5'-GGGTTT-3' on the ss2 strand resulted in loss of nm23- β binding. The RE-1 sequence is not pyrimidine rich (18 out of 40 bp) and the mutagenesis findings, coupled with the direct competition for YB-1 binding, are most consistent with a sequence dependency for nm23- β binding. The mutation studies are compatible, however, with a requirement for pyrimidine nucleotides within the larger recognition sequence. The telomeric repeat sequence TTAGGG has been shown [16] to bind recombinant glutathione S-transferase (GST)-nm23-H2, whereas recombinant GST-nm23-H1 did not bind under the conditions used. The differences in binding to these closely related, but inverted sequences (5'-GGGTTT-3' versus 5'-TTAGGG-3'), are possibly due to the consequence of the types of recombinant proteins used (GST versus hexahistidine fusion proteins), the orientation or a requirement for the appropriate nucleotide sequence matrix surrounding the

core recognition motifs. We note that recombinant rat nm23- α had little or no RE-1 binding activity and that transfection with nm23- α cDNA did not suppress luciferase activity, suggesting that the results detailed in the present study are restricted to nm23- β .

Although gelatinase A is a recognized mediator of the metastatic process, these studies should not be taken to suggest that the sole mode of nm23- β metastatic suppression occurs through the blockade of gelatinase A transcription. For example, earlier studies of nm23- β suppression used breast carcinoma MDA-MB-435 cells [20]. This cell type does not synthesize gelatinase A, but does produce significant quantities of a second prometastatic metalloproteinase membrane-type-1 MMP (MT1-MMP) [32]. In transfection studies using MT1-MMP promoter/luciferase constructs, no evidence for suppression of MT1-MMP transfection by nm23- β was found (D. H. Lovett, unpublished work). Given the multifaceted nature of the metastatic process and the complex biochemical changes characteristic of tumour cells, it would be safer to consider suppression of gelatinase A transcription by nm23- β as but one component of many that determine the actual metastatic capabilities of an individual cell type.

Finally, the competitive interaction of nm23- β with YB-1 for binding to the RE-1 sequence suggests that similar competitive events may occur in the regulatory regions of other genes known, or suspected, to be regulated by YB-1. These genes include thymidine kinase, proliferating cell nuclear antigen ('PCNA'), the epidermal growth factor receptor and DNA polymerase- α [33–35]. These are key growth regulatory genes and the potential modulation of their transcription by the abundant nm23 proteins offers an intriguing extension of the biological activities of this unusual gene family.

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