Mammalian DNA cytosine-5 methyltransferase interacts with p23 protein

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Abstract In higher eukaryotic genomes, methylated cytosine residues (m^5C) are distributed in heritable, cell-type-specific patterns, which are believed to be involved in the control of gene expression, developmental regulation and genomic imprinting. These methylation patterns are established and maintained by DNA cytosine-5 methyltransferase (MTase), a ~1500 amino acid enzyme containing a regulatory N-terminal domain and a catalytic C-terminal domain. The mechanism responsible for targeting MTase to particular genes is poorly understood and might possibly involve interactions with other proteins. In an effort to identify proteins that interact with the mammalian MTase, we used the yeast two-hybrid system with several different MTase domains as baits. Here we report an interaction between the C-terminal catalytic domain of the MTase and p23, a protein previously reported to associate with the progesterone receptor (PR) complex.

Key words: Mammalian DNA cytosine-5 methyltransferase; p23 protein; Yeast two-hybrid system; Protein–protein interaction

1. Introduction

Methylated cytosine residues (m^5C) are distributed in heritable, tissue-type-specific manners in higher eukaryotic genomes. These cytosine methylation patterns have been implicated in the control of gene transcription, development and genomic imprinting in mammals [1–5]. DNA cytosine-5 MTase is the enzyme believed to be primarily responsible for establishing and propagating the methylation patterns through cell generations [6]. Consistent with this notion, targeted disruption of the *MTase* gene in the mouse results in embryonic lethality [7]. The mammalian MTase protein contains an N-terminal domain of about 1000 amino acids, which has no counterpart in the bacterial enzymes, in addition to a C-terminal 500 amino acid domain that is closely related to bacterial cytosine-5 MTases [8]. Contained within the N-terminal domain are several putative sub-domains, including a Cys-rich region [9] and a targeting sequence that mediates the association of DNA MTase with the replication apparatus during S-phase [10]. The C-terminal domain contains residues that are essential for sequence specific recognition of DNA and for catalysis of methyl transfer. Mammalian MTase operates preferentially on hemimethylated DNA, thus ensuring the clonal propagation of cell-type-specific methylation patterns during cell division. Proteolysis, which leaves the C-terminal domain intact but cleaves the N-terminal domain into smaller fragments, greatly stimulates the enzyme's de novo activity on unmethylated substrates [11], suggesting that N-terminal domain can modulate the specificity of the C-terminal catalytic domain. The mechanism underlying this regulatory effect is not known, nor is it understood how the enzyme is targeted to certain genes during embryonic development.

One possible mechanism for recruitment of MTase to certain genes might involve direct interactions with other proteins, by analogy to the process of transcription initiation in eukaryotes [12,13]. A well-defined segment of the N-terminal domain is responsible for targeting the MTase to replication foci, most likely through protein–protein interactions. To identify potential interacting proteins of MTase, we utilized two-hybrid screening assays in yeast [14,15]. Several segments of the MTase N-terminal domain, including the replication targeting domain, themselves activate transcription when fused to the LexA DNA-binding domain, which precluded two-hybrid screening using these segments. No interacting proteins for the Cys-rich region were identified. However, the C-terminal catalytic domain of the MTase was found to associate with the p23 protein, best known for its association with the cytoplasmic form of the progesterone receptor. In vitro binding assays confirmed that p23 and MTase interact specifically, and the results of co-immunoprecipitation experiments provide evidence that the two can associate in vivo.

2. Materials and methods

2.1. Plasmids

Four different regions of human MTase [16] were PCR-amplified by *pfu* polymerase (Stratagene) from pBSHMT (gift from Dr. S. Baylin, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA) using primers that included on their ends either an *Eco*RI (N-terminal primer) or *Sal*I (C-terminal primer) site. The PCR product was restricted with *Eco*RI and *Sal*I and cloned into the vector pEG202 [15] to generate baits having residues 1–147 of the LexA DNA-binding domain fused at its C-terminus to various regions of MTase: pLexA-hMT(207–455), pLexA-hMT(529–607), pLexA-hMT(608–1004) and pLexA-hMT(999–1495):C1105A (contains an active site Cys to Ala mutation). Both pRFHM1 (LexA DNA-binding domain fused to *Drosophila* bicoid protein) and a HeLa cDNA library tagged with the B42 activation domain were kindly provided by Dr. R. Brent (Harvard Medical School, Boston, MA 02114, USA).

The full-length mouse MTase cDNA (gift from Dr. T. Bestor, Columbia University, New York, USA) was equipped with 6×His and Myc epitope (EQKLISEEDL) tags at the N-terminus, cloned into the mammalian expression vector pEVRF0 [17] between *Kpn*I and *Xba*I sites to generate p(His)$_6$Myc-mMT. The p23 protein was expressed as a glutathione *S*-transferase (GST) fusion protein using a Pharmacia pGEX-5X vector. All constructs were verified by dideoxy sequencing.

2.2. Two-hybrid assays

The yeast selection strain EGY48 and *lacZ* reporter were used as described [18,19]. Cells were transformed with both LexA DNA-binding domain fusion baits and B42 activation domain fusion library by

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Abbreviations: MTase, mammalian DNA cytosine-5 methyltransferase; GST, glutathione *S*-transferase; X-Gal, 5-bromo-4-chloro-2-indolyl β-D-galactoside; MEL, murine erythroleukemia cell; PR, progesterone receptor

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the lithium acetate method. The transformation mixtures were plated on appropriate selective synthetic media. To represent adequately the complexity of the cDNA library, we obtained 5×10^6 primary transformants. After 3 days at 30°C, these transformants were harvested and mixed thoroughly as a pool. About 5×10^7 colony forming units (cfu) from this pool were induced with galactose to drive the library protein expression and then plated on media lacking leucine for *LEU2* reporter selection. All the colonies appearing after 4 days were subject to 5-bromo-4-chloro-2-indolyl β-D-galactoside (X-Gal) filter assays for *lacZ* reporter expression. Only those *LEU2* and *lacZ* double-positive colonies were picked up and assayed again to confirm their galactose-dependent expressions of *LEU2* and *lacZ* reporters.

2.3. GST fusion protein binding assays

pGEX5X-p23 was transformed into *E. coli* strain BL21 and a mid-log phase cell culture was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) and grown for 4 h at 37°C. Cell lysates were then loaded on a glutathione-agarose column which was subsequently washed 3 times with PBS. MEL cell nuclear extracts or 50% pure Myc-tagged mouse MTase were passed through minicolumns prepacked with GST-p23 bound resin or GST bound resin. These resins were then washed 3 times with 1 of ml buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol, and subsequently suspended in SDS sample buffer and analyzed by immunoblotting with anti-pATH52 or anti-Myc antibody 9E10.

2.4. Co-immunoprecipitation

Nuclear extracts of Friend murine erythroleukemia (MEL) cells were made following procedures described by Roeder et al. [20]. The polyclonal rabbit anti-mouse MTase antibody anti-pATH52 [11] was a gift from Dr. T. Bestor. The monoclonal anti-p23 antibodies JJ3 and JJ5 [21] were generously provided by Dr. D. Toft (Mayo Graduate School, Rochester, MN 55905, USA). MEL nuclear extracts (1 ml) were precleared with 20 μl of preimmune rabbit serum or an unrelated monoclonal antibody followed by incubation with 25 μl of protein A positive *S. aureus* cell pellet. After centrifugation, 4 μl of anti-pATH52 or 15 μl of JJ3 was added to the supernatant followed by shaking at 4°C for 1 h. Mock immunoprecipitation controls were run without addition of antibody. Fifty microliters of slurry of 50% protein A-Sepharose resin (Pharmacia) was then added to the mixture followed by shaking for another 1 h. The resin was pelleted and washed 4 times with washing buffer (20 mM Hepes, pH 7.9, 150 mM NaCl, 0.1% Triton X-100) with subsequent resuspension in SDS buffer. The samples were denatured by boiling for 5 min and analyzed on SDS-PAGE gel. Proteins were visualized by silver staining and/or Western blot analysis.

2.5. Mammalian expression and purification of Myc-tagged mouse MTase

COS-7 cells were transfected with p(His)$_6$Myc-mMT using DEAE-DEXTRAN methods [10]. After 48 h, the COS cells were harvested by trypsination and lysed in 5 vol of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.3% Triton X-100, 0.4 M NaCl, 1 mM DTT, 1 mM PMSF, 2 μg/ml of each aprotinin, pepstatin A, leupeptin, benzamidine). The lysate was incubated with Ni^{2+} NTA-resin at 4°C followed by elution of the Myc-tagged mouse MTase with a linear gradient of imidazole (0.8–300 mM). The recombinant MTase was purified to 50% homogeneity upon elution with 40–80 mM imidazole.

3. Results

3.1. Two regions of human MTase can be used as baits

Constructs containing four different regions of the fused to the DNA-binding domain of LexA were generated as potential 'baits' for two-hybrid screening (Fig. 1). The MTase regions chosen for study are the replication-targeting sequence (207–455); a Cys-rich motif (529–607) related to that in the human trithorax homologue, HRX/ALL-1 [9]; a large region of unknown function located between the Cys-rich and catalytic domain (608–1004); and the entire C-terminal catalytic domain (999–1495). To avoid complexity that might arise through the methylation of yeast DNA by fusing the MTase catalytic domain to LexA, we mutated an essential catalytic residue, Cys1105, to Ala. In the context of the full-length MTase protein, the C1105A mutation abrogates catalysis but leaves intact the ability of the protein to bind DNA (X.Z. and G.L.V., unpublished results). Similar observations have been reported with the bacterial homologues of the human MTase [22,23]. Baits I–IV were first tested for their ability to activate the transcription of *lacZ* and *LEU2* reporter constructs, each driven by multiple LexA operators. As shown in Fig. 1, baits I and III by themselves strongly activated reporter gene transcription, and thus were unsuitable for use in two-hybrid screening.

On the other hand, baits II and IV showed no detectable reporter gene activation. Repression assays confirmed that the bait II and IV fusion proteins enter the yeast nucleus and bind

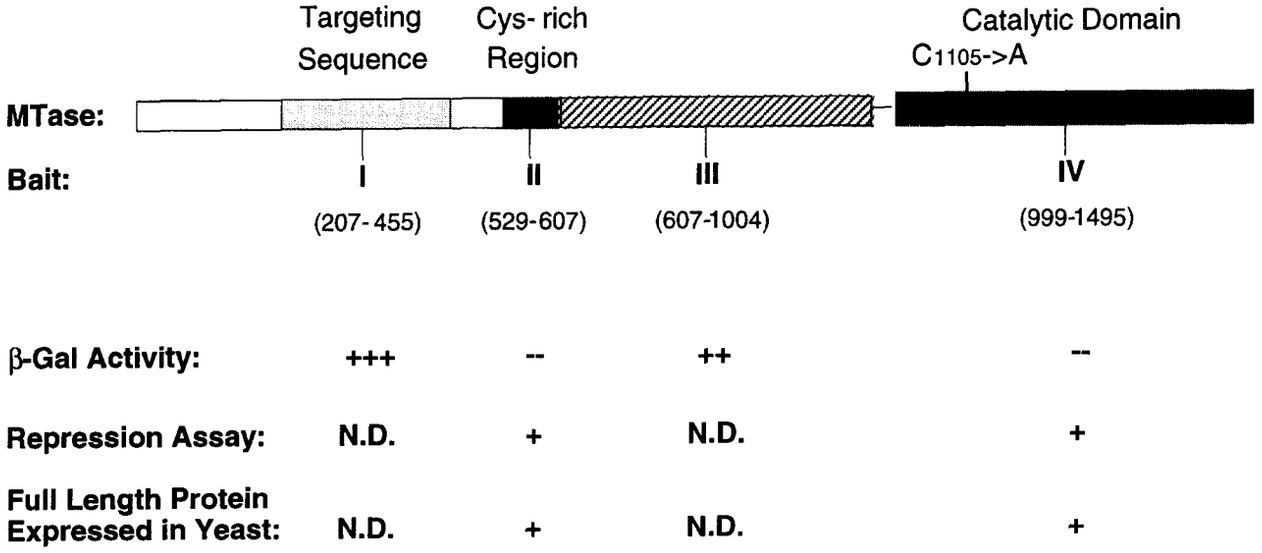

Fig. 1. Bait constructs and activity tests. Baits I and III activated reporter gene transcription and hence were not studied further. Baits II and IV exhibited the least levels of β-galactosidase activity and therefore were analyzed in repression assays. A (+) repression assay result indicates that the bait construct is capable of entering the yeast nucleus and binding to the reporter gene. Both baits II and IV were expressed in yeast as full-length fusion proteins, as determined by Western blotting using an anti-LexA antibody (data not shown). N.D.: not determined.

Table 1
β-Galactosidase activities of positive clones from two hybrid screens

B42 activation	LexA DNA-binding domain fusion			
comain fusion	hMTase(529-607)	hMTase(999-1495)	Bicoid	ORF (aa)
Clone 1	–	++	+/–	177
Clone 2	–	++	+/–	175
Clone 3	–	++	+/–	177

The yeast reporter strain EGY 48 was transformed with the indicated LexA DNA-binding domain and B42 activation domain fusion plasmids and plated on selective synthetic medium. The expression of β-galactosidase was monitored by using the assays described in Section 2 and scored on a scale of – to ++ according to the rate at which blue color developed. The positive clones were sequenced to identify the open reading frames (ORF) of the non-LexA derived portions of the fusion constructs.

to LexA operators (Fig. 1). Immunoblotting of yeast cell extracts using a polyclonal antibody directed against LexA verified the expression of the full-length LexA-fusion baits II and IV proteins. Therefore, baits II and IV were deemed suitable for use in two-hybrid screening.

3 2. Recovery of the p23 from a two-hybrid screen

We used the yeast two-hybrid system as modified by Brent and colleagues (interaction trap) [15,19] to identify proteins interacting with domains II and IV of the human MTase. Bait constructs II and IV were separately used to screen a human HeLa cDNA library in which the inserts were expressed as fusions to the *E. coli* B42 activation domain [15]. A yeast strain bearing integrated *LEU2* and extrachromosomal *lacZ* reporters was transformed with either the bait II or IV-encoding plasmid and the B42 fusion cDNA library to generate ~5×10^6 independent transformants. Cells harboring candidate MTase interactors were identified by their ability to grow on media lacking leucine and to turn blue on X-Gal impregnated filters upon induction by galactose. The roughly 100 colonies from baits II and IV that passed this initial selection were patched to glucose media to silence the expression of library proteins, then replica-plated to four different selection media (Leu^-/Gal, Leu^-/Glu, X-Gal/Gal, X-Gal/Glu) to test whether the activation of *LEU2* and *lacZ* reporter genes were dependent on galactose induced library protein expressions. Finally, positive library plasmids were isolated from 36 bait II colonies and 25 bait IV colonies, retransformed into the parent yeast reporter strain with either the original bait plasmid or unrelated bait plasmid pRFHM1 (LexA-bicoid fusion construct). In this tertiary screen, none of the library clones screened using bait II scored positive for plasmid linkage of reporter gene activation. However, 10 of the 25 library clones screened using bait IV were able to activate reporter gene activation in the presence of bait IV; none of these strongly activated the reporters in the presence of bait II or the control LexA–bicoid fusion construct. Interestingly, the inserts of all 10 plasmids had a similar size (~1.5 kb) and yielded almost identical restriction patterns upon cleavage by *Alu*I or *Hae*III (data not shown). Sequencing of the inserts in 3 out of 10 clones revealed that they possess a virtually identical open reading frame of around 175 amino acids. A search against the GenBank nucleic acid sequence database indicated that the clones are identical to the human *p23* cDNA[21]. Sequences 45 to 51 nucleotides upstream of the p23 start codon were also included in these clones, resulting the addition of 15 to 17 residues between the LexA DNA-binding domain and the full-length p23 protein (Table 1).

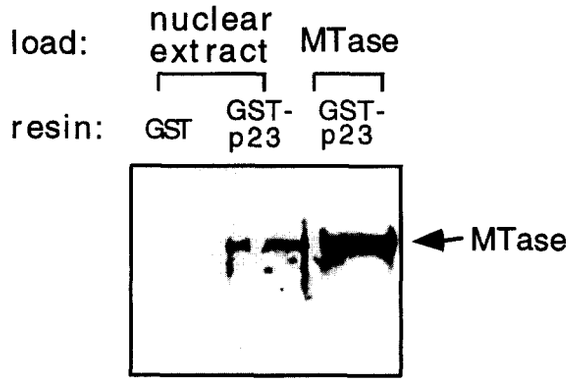

Fig. 2. p23 specific binding by MTase in vitro. Partially purified Myc-tagged MTase or MEL nuclear extract were loaded to glutathione-agarose columns containing either bound GST or GST-p23. After washing, the resin bound proteins were fractionated by SDS-PAGE, blotted and probed for the presence of MTase using an anti-MTase antibody (anti-pATH52).

3.3. Binding of the MTase to p23 in vitro

p23, so named for its apparent molecular weight on SDS-PAGE gels, is an ~18 kDa protein of wide tissue distribution, which is highly conserved throughout eukaryotes. p23 was first observed as a component of the cytoplasmic form of the avian progesterone receptor (PR) complex [24], but it has also been found associated with the 90 kDa heat shock protein, hsp90 [25].

To determine if MTase and p23 interact directly independent of any yeast bridging proteins, a GST-fusion protein was expressed containing the full-length p23. The bacterially overproduced GST-p23 or GST control were purified on glutathione agarose and then incubated with MEL nuclear extract or purified mouse MTase. Bound MTase were detected by immunoblotting with anti-MTase (Fig. 2). As shown, GST-p23 could bind to both purified mouse MTase and endogenous MTase in MEL nuclear extract. As a control, GST alone did not bind to MTase detectably. This in vitro binding assay provides evidence that MTase and p23 can interact directly.

3.4. Association of MTase with p23 in MEL cells

To compare the tissue distribution of p23 with that of the MTase, we carried out Western blot analysis using nuclear and cytoplasmic extracts of murine erythroleukemia (MEL) cells (Fig. 3). As previously observed [10], the MTase is localized primarily in the nucleus (Fig. 3A). p23 is also found in the nucleus, but is equally distributed in the cytoplasm of MEL cells (Fig. 3B). To assess the interaction of p23 and

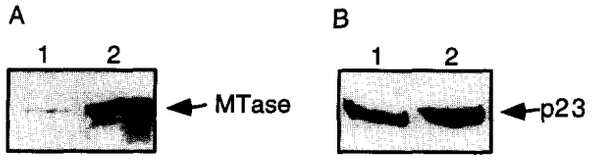

Fig. 3. Distribution of MTase and p23 in MEL cytosol and nuclear extract. A: MTase is a nuclear protein. MEL cytosol (C) and nuclear extract (N) each 10 μl was run on a 5% SDS-PAGE gel, transferred to nitrocellulose membrane and blotted with mouse MTase antiserum anti-pATH52. B: Similar levels of p23 were detected in MEL cytosol and nuclear extract. Equal amount (10 μl) of MEL cytosol and nuclear extract from the same preparation as above were blotted with p23 antibody JJ3.

the MTase in vivo, we used a polyclonal antibody (anti-pATH52) that targets the N-terminal domain of the mouse MTase to immunoprecipitate MEL cell nuclear extracts, then analyzed the interacting proteins by SDS-PAGE (Fig. 4A) and Western blotting using monoclonal anti-p23 antibodies (Fig. 4B). Anti-pATH52 precipitated a band having a molecular mass of 23 kDa (Fig. 4A, lane 2), which was absent from the mock precipitation without anti-pATH52 (lane 1). The 23 kDa species immunoprecipitated using an anti-MTase antibody had the same mobility as that of p23 in whole MEL nuclear extracts, as judged by Western blot analysis (Fig. 4B). Reciprocal immunoprecipitation with anti-p23 and Western blotting using anti-MTase also indicated the two proteins interact in MEL cell extracts (data not shown). A Myc-tagged mouse MTase was overproduced in COS cells and partially purified, this was then added to the MEL nuclear extract and precipitated using a monoclonal anti-Myc antibody 9E10 (Fig. 4C). Again, the 23 kDa band appeared only in the 9E10 precipitation lane but not in the mock lane without 9E10. These results indicate that the MTase and p23 interact specifically in MEL cell extracts.

4. Discussion

Here we have used the yeast two-hybrid assay to screen a fusion cDNA library for proteins that interact with the human MTase protein. Two regions of the MTase, the replication-targeting domain (bait I) and the region immediately N-terminal to the catalytic domain (bait III), themselves activate reporter gene transcription when fused to the DNA-binding domain of LexA. We do not ascribe any biological significance to this finding, as many sequences possess the ability to stimulate transcription when linked to a DNA-binding domain [26]. In particular, sequences rich in acidic residues can often function as activation domains; regions I and III of the human MTase have pIs of 4.3 and 5.7, respectively. On the other hand, regions II and IV of the MTase are more basic (pIs of 9.6 and 9.9, respectively), and lack the ability to activate transcription.

Two-hybrid screens using the Cys-rich domain of the MTase (bait II) failed to identify any interacting proteins. When a mutationally inactivated form of the C-terminal catalytic MTase domain was used as bait (bait IV), 10 positive interacting clones were obtained from the B42-cDNA fusion library after tertiary screening. All of these clones encoded the same B42 fusion partner, identified as the full-length product of the human *p23* gene. The repeated isolation of a single strongly interacting partner from a library having a complexity of $\sim 10^6$ argues that the human MTase C-terminal domain is not simply 'sticky', and suggests the MTase and p23 bind one another specifically in the yeast cell, where high concentrations of non-specific competitor proteins are present. The results of in vitro binding assays demonstrate that p23 is able to interact directly with the full-length MTase.

At present, the functional role of the p23–MTase interaction is uncertain. p23 was originally cloned as a protein associating with the cytoplasmic ('untransformed') form of the progesterone receptor, in a complex containing several addi-

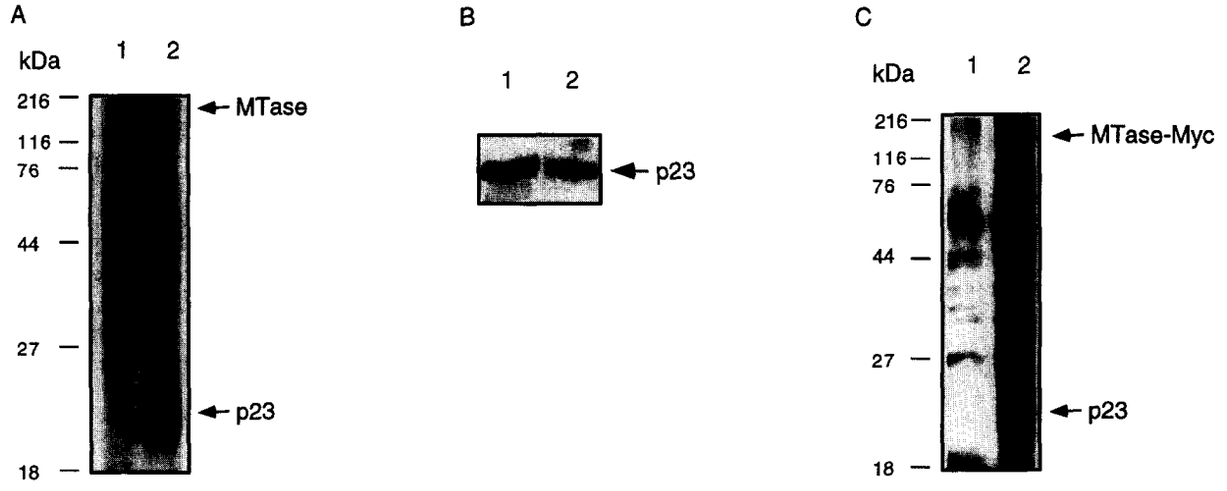

Fig. 4. Association of mouse MTase with p23 protein in MEL cell extracts. A: Immunoprecipitation of p23 with anti-MTase antibody (anti-pATH52). The complexes were analyzed on a 12% gel and silver-stained. Lane 1, mock immunoprecipitation without anti-pATH52; lane 2, immune isolation from MEL nuclear extracts with anti-pATH52. Positions of molecular weight markers are shown on the left. B: The p23 kDa species precipitated by an anti-MTase antibody is detected by anti-p23 (JJ3). Lane 1, control lane showing immunoblotting of p23 from MEL cell nuclear extracts; lane 2, immunoblotting of p23 from the immunoprecipitated proteins isolated by anti-pATH52. C: Monoclonal antibody 9E10 against Myc immunoprecipitates p23 from MEL nuclear extracts supplemented with Myc-tagged MTase. Lane 1, mock immunoprecipitation without antibody 9E10; lane 2, immune isolation with 9E10. Positions of molecular weight markers are shown on the left.

tional proteins [21]. It has been suggested that p23 is a component of several other cytoplasmic complexes of steroid receptor complexes [27,28]. p23 may also form complexes with the hsp90 in the absence of steroid hormone receptors; however, unlike heat shock protein, p23 is not induced thermally [21,29]. p23 is known to be phosphorylated [21] in resting cells, but the role of the phosphorylation, if any, remains to be determined. The function of p23 in the complexes it forms, or in the cell as a whole, is unknown. Moreover, p23 lacks discernible sequence similarity to any known proteins. Despite this lack of information, it seems likely that p23 fulfills an essential functional role, as the protein is highly conserved throughout eukaryotes, and its sequence from birds through mammals is close to invariant.

The finding that p23 interacts with the C-terminal domain of the MTase suggests that p23 might modulate the catalytic activity of the MTase. However, we were unable to detect any effect of GST-p23 on the rate of transfer of radioactivity from [*methyl*-^{3}H]S-adenosyl methionine to poly(dI-dC) [30,31], even when GST-p23 was present at relatively high concentrations (data not shown). Because the C-terminal domain is so large ($\sim$60 kDa), it can readily be envisioned to present a binding surface to p23 that lies outside the region involved in catalysis of the methyl transfer reaction.

Prior to the present study, all known complexes of p23 were localized in the cytoplasm. Because the MTase is present almost exclusively in the nucleus, its interaction with p23 would most likely take place in the nucleus. Indeed, we found that in MEL cells, p23 partitions almost equally between the nucleus and cytoplasm. Because p23 is known to associate with hsp90, which in turn binds actin filaments [32,33], it is tempting to speculate that p23 might recruit the MTase to the nuclear matrix via hsp90.

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