MacroH2A1.2 binds the nuclear protein Spop

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Abstract

X-chromosome inactivation is a phenomenon by which one of the two X chromosomes in somatic cells of female mammals is inactivated for life. The inactivated X chromosomes are covered with Xist (X-inactive specific transcript) RNA, and also enriched with the histone H2A variant, macroH2A1.2.The N-terminal one-third of macroH2A1.2 is homologous to core histone H2A, but the function of the C-terminal two-thirds, which contains a basic, putative leucine zipper domain, remains unknown.In this study, we tried analyzing protein–protein interaction with a yeast two-hybrid system to interact with the nonhistone region of mouse macroH2A1.2. The results showed that macroH2A1.2 interacts with mouse nuclear speckled type protein Spop. The Spop protein has a unique composition: an N-terminal MATH, and a C-terminal BTB/POZ domain. Further binding domain mapping in a glutathione-S-transferase (GST) pull-down experiment revealed that macroH2A1.2 binds the MATH domain of Spop, which in turn binds to the putative leucine zipper domain of macroH2A1.2.
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1. Introduction

Macrohistone H2A (macroH2A) is a histone H2A variant. The N-terminal one third of macroH2A is homologous to histone H2A, but the remaining two-thirds is a novel polypeptide with unknown function [1,2].

The recently demonstrated subtype of macroH2A1, macroH2A1.2 localizes on the inactive X chromosomes of female mammals [3]. The specific association between macroH2A1.2 histone and the inactive X chromosome suggests that macroH2A may function in the inactivation process. The association between macroH2A1.2 and the inactive X chromosome depends on the continued presence of the X-inactive specific transcript (Xist), the large untranslated RNA that associated in *cis* along the length of the inactive X [4]. Although macroH2A1.2 has been

reported to be physically associated with Xist RNA, no specific role of macroH2A1.2 in X inactivation has yet been identified.

X-chromosome inactivation occurs in three steps: initiation, spreading, and maintenance [5], and a noncoding large RNA, Xist, is involved in each step [5]. The histone-H2A homologous region of macroH2A plays a crucial role in the association between macroH2A molecules and inactive X chromosomes according to the results of chromatin immunoprecipitation using anti-macroH2A antibody followed by reverse transcription-coupled polymerase chain reaction (RT-PCR) with an extract of female cells [6]. A recent study has shown that the association between macroH2A and the inactive X, which seems to depend on the histone H2A domain of the macroH2A molecule rather than the nonhistone domain, may reflect the higher density of several histones on the inactive X [7]. Study of its genomic structure has revealed that the gene at the macroH2A locus contains 10 exons [8]. The macroH2A locus produces two protein isoforms, 1.1 and 1.2, by alternative splicing. Exon 6 has been found to be a macroH2A1.2-specific exon, while exon 7 is macroH2A1.1-specific. MacroH2A1.2 is preferentially associated with the inactive X chromosome since exon 6 contains a putative leucine zipper motif [8].

In order to understand the function of macroH2A1.2 on inactive X chromosomes, we used a yeast two-hybrid screen to search for a protein module(s) that is(are) associated with the nonhistone H2A domain of macroH2A1.2.

2. Methods

2.1. Plasmid

We amplified either full-length cDNA sequences or selected regions of their sequences by polymerase chain reaction (PCR) using restriction enzyme-site-tagged primers and AmpliTaq DNA polymerase (PE Applied Biosystems).

The PCR products were digested with appropriate enzymes and usually cloned into a pcDNA vector (Invitrogen) and sequenced for PCR errors (ABI Sequencer).

We cloned various domains of mouse Spop or mouse macroH2A1.2 into the pGEX vector (Amersham Pharmacia Biotech) by PCR using appropriate sets of primers, and also cloned mistake-free products in-frame into the appropriate vector(s).

2.2. Two-hybrid screening

Two-hybrid screening in yeast was performed with the system of Gyunis et al. [9]. Briefly, the yeast strain used was EGY48/LacZ, which carries two reporters whose expression is regulated by LexA-responsive promoters: a chromosomally integrated LEU2 reporter gene (LexA:LEU2) and the 2μLacZ reporter plasmid pSH18-34. pEG202-macroH2A (aa110–372) was used as bait in EGY48/LacZ to screen the mouse embryo Day 19 pJG4-5 prey library (OriGene MD). A total of 2×10^6 yeast cotransformants were selected for galactose-induced reporter-dependent leucine prototrophy. Prey plasmids were rescued from positive clones and retransformed into EGY48/LacZ strains expressing pEG202-macroH2A. Cotransformants were reselected for reporter-dependent leucine prototrophy and β-galactosidase production, and positive clones were sequenced.

2.3. Glutathione-S-transferase (GST) pull-downs of in vitro translated proteins

GST and GST fusion proteins were expressed in *E. coli* DH5α by using the pGEX (Amersham Pharmacia) vector system. Bacteria were induced with 0.2 mM IPTG for 4 h at 37 °C, and recombinant proteins were purified with glutathione-Sepharose beads and analyzed on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to normalize protein amounts. Equivalent amounts of GST fusion proteins were incubated with [^{35}S]methionine-labeled mouse macroH2A1.2 or Spop proteins, produced by a T7-TNT-coupled transcription/translation system (Promega, WI) in 200 μl GST buffer(20 mM Tris–HCl pH 8, 100 mM NaCl, 1 mM EDTA) added with 0.1%

NP40, a proteinase inhibitor cocktail (Roche). After 1-h incubation on ice, the beads were washed four times with GST buffer. The bound proteins were eluted with SDS sample buffer and analyzed on SDS-PAGE followed by autoradiography [10].

2.4. Immunofluorescence analysis

C127 cells were plated onto coverslips and transfected with pEGFP-Spop using the lipofectamine PLUS regent (Gibco BRL), according to the manufacturer's instructions. After 24 h, the cells were fixed with a paraformaldehyde solution (4% paraformaldehyde in PBS) for 15 min followed by a 5-min incubation in 0.2% Triton X100/PBS. The cells were subsequently blocked with 5% skim milk (Difco, MI) in $2 \times$ SSC for 1 h. After blocking, anti-macroH2A1.2 rabbit antibody (Upstate Biotech, NY) was added to the cells and incubated at room temperature for 3 h. After washing, the coverslips were incubated with Texas red conjugated anti-rabbit goat antibody (Molecular Probes) for 1 h at room temperature and finally mounted in Vectashield anti-fade (Vector Laboratories, CA) containing DAPI.

2.5. Confocal microscopy

Samples were analyzed with the laser confocal scanning microscope IX81 (Olympus). UV–Ar (351 nm), Ar (488 nm), and HeNe (543 nm) lasers were used to excite DAPI, enhanced green fluorescent protein (EGFP), Texas red fluorescence, respectively. The lens was an Olympus Apo40XWLSM/UV objective.

2.6. EGFP-Spop

The full coding sequence from Spop was PCR-amplified with primers incorporating *Hin*dIII and *Sal*I restriction enzyme recognition sites and subcloned into pEGFP-C1 (Clontech) vector.

3. Results

3.1. Isolation of mouse macroH2A1.2 cDNA

The mouse macroH2A-specific nonH2A region was amplified by PCR using rat macroH2A-specific primers: forward, 5′ CCA AAA AGG CCA AGT CTC CAT 3′, and reverse, 5′ ACA TGT GCT GGC TCT AGG GAA 3′, and random primed cDNA from mouse MC12 cell as the template. PCR products from 30 cycles of amplification at 95 °C 1 min and 58 °C 5 min were cloned into pGEM-T Easy Vector (Promega) to generate a mouse macroH2A1 clone referred to as pGEM-Macro.

Since the pGEM-Macro had a 6-bp deletion compared with the rat macroH2A sequence because of a PCR error, we tried to isolate a complete mouse macroH2A cDNA clone

from a mouse brain cDNA library (Stratagene, CA) with the 0.7 kb-insert of pGEM-Macro as a probe. The clones picked were identified by their insert-sequences, and one of the clones obtained, Ma-8, was shown to have the entire sequence of mouse macroH2A1.2.

3.2. Isolation of macroH2A1.2 interacting proteins

The macrohistone H2A1.2 has been reported to concentrate on the inactive X chromosomes of female mammals. To better understand the role of macroH2A1.2, we used a yeast two-hybrid approach to identify interacting proteins. Fusion between the LexA DNA binding protein and amino acids 110–372 of the macroH2A protein (Fig. 1A) was used as bait to screen cDNA from mouse embryo Day 19 DNA inserted into an expression cassette consisting of the SV40 nuclear localization sequence, the acid blob B42, and the hemagglutinin (HA) epitope tag under the control of the Gal1 inducible promoter.

Four independent clones were isolated from the approximately 2×10^6 transformants on the basis of their ability to interact with non-H2A region of macroH2A1.2. The screening led to the identification of a sequence derived from a mouse SPOP (Spop) cDNA coding amino acids 2–374 (Fig. 1.B) as a possible macroH2A1.2 interacting partner.

3.3. In vitro interaction between Spop and macroH2A1.2

An independent assay was used to confirm the Spop–macroH2A1.2 interaction detected in yeast. The Spop product was expressed in fusion with GST, immobilized on glutathione–agarose beads, and tested for interaction with macroH2A1.2 produced in vitro. MacroH2A1.2 was specifically retained on beads covered with the GST–Spop fusion product, compared to background binding to GST alone (Fig. 2A). GST–Spop pulled down macroH2A1.2, but GST alone did not pull down any appreciable amount of mac-

Fig. 2. MacroH2A1.2 interacts with the nuclear protein Spop in vitro. The Spop consisting of an amino-terminal MATH (GST–MATH) domain or the carboxyl terminal BTB/POZ (GST–POZ) domain fused with GST was used in GST pull-down experiments with [^{35}S]-labeled in vitro translated non-H2A region of macroH2A1.2. Whole GST–Spop fusion proteins and GST alone were used as controls. The "Input" lane represents 10% of the probe used for the interactions. Molecular mass standards in kDa are shown.
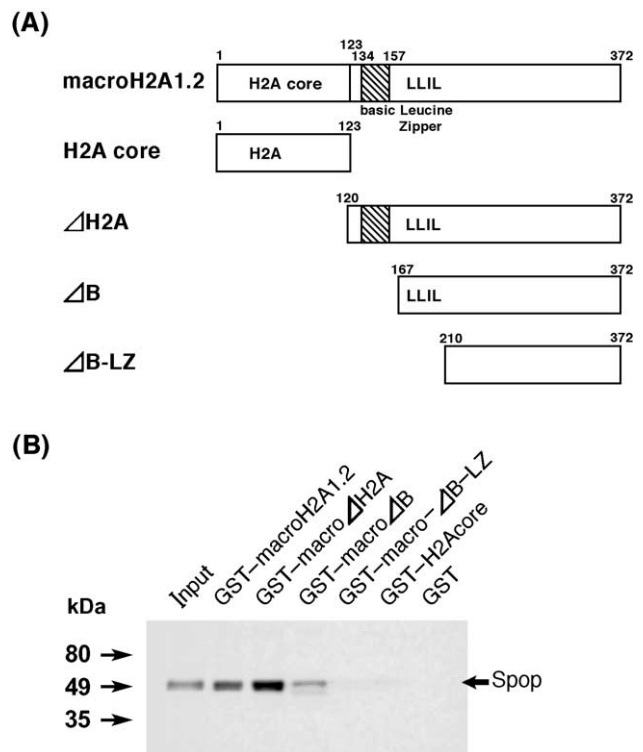
Fig. 3. Spop protein interacts with the GST-fusion proteins of macroH2A1.2 truncation constructs. (A) Schematic representation of macroH2A1.2 truncation constructs. (macroH2A1.2) macroH2A1.2; (H2A) histone H2A homologous region aa1–123; (ΔH2A) nonhistone region aa120–372; (ΔB) nonhistone region aa167–372 deletion of basic domain; (ΔB–LZ) nonhistone region aa210–372 deletion of basic domain and putative leucine zipper motif (LLIL). (B) Spop protein interacts with the GST-fusion proteins of macroH2A1.2 truncation constructs. In vitro pull-down experiments were performed with ^{35}S-labeled, in vitro-translated Spop. The macroH2A1.2 full-length and truncation derivatives shown in (A) were expressed as GST-fusion proteins. The "Input" lane represents 10% of the probe used for the interactions.
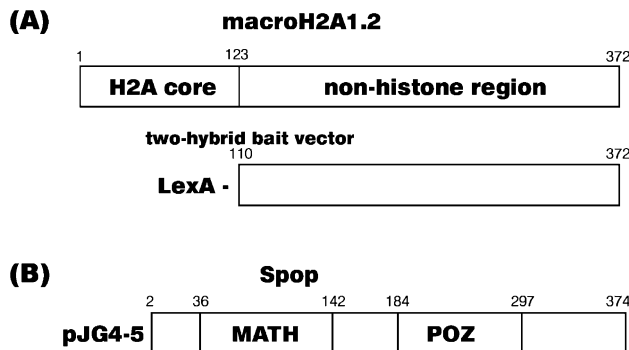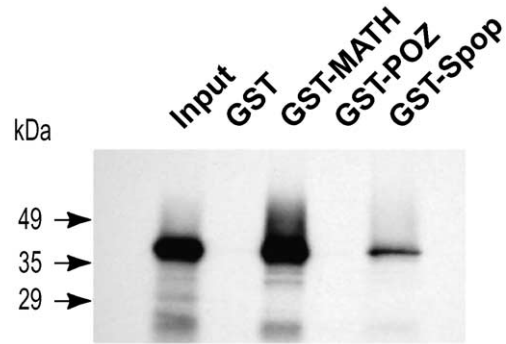
Fig. 1. MacroH2A1.2 binds the nuclear protein Spop in a yeast two-hybrid screen. (A) Schematic representation of macroH2A1.2. The N-terminal one-third is homologous to core histone H2A, and the remaining C-terminus two-thirds is macroH2A1.2 specific region. (B) Schematic representation of Spop. The N-terminal MATH domain and the C-terminal BTB/POZ domain are shown.

roH2A1.2, indicating specific interactions in vitro between GST–Spop and macroH2A1.2.

3.4. Mapping the binding domain of Spop and macroH2A1.2

The Spop protein consists of two domains, the MATH domain and the BTB/POZ domain (Fig. 1B). Then, we investigated which domains of Spop interacted with macroH2A1.2. We constructed GST fusion proteins that contained either the MATH or BTB/POZ domain of Spop protein and assayed them by GST pull-down experiments as above. We observed that the MATH domain of Spop interacts with macroH2A1.2 (Fig. 2B), and the GST pull-down experiments showed that the Spop MATH domain (aa 91–113) is capable of interacting with macroH2A1.2. The macroH2A1.2 protein consists of an N-terminal third that is homologous to core histone H2A and a C-terminal two third novel protein containing a basic region and putative leucine zipper (Fig. 3A). To identify the part of macroH2A1.2 involved, we constructed GST-fusion proteins of various truncated forms of macroH2A1.2 (Fig. 3A) and assayed them by GST pull-down experiments. The difference

between macroH2A1.1 and 1.2 lies in whether a putative leucine zipper is present or not, and the macroH2A1.2 protein contains a putative leucine zipper. Our results confirmed that this leucine zipper is important to the binding between macroH2A1.2 and Spop (Fig. 3B).

3.5. In vivo interaction between Spop and macroH2A1.2

To investigate the in vivo interaction of Spop with macroH2A1.2, we introduced an expression construct of EGFP–Spop into C127 mouse cells, and the transiently expressed fluorescent tagged protein and endogenous macroH2A1.2 protein were observed.

The EGFP-tagged Spop expressed a discrete speckled pattern in nuclei (Fig. 4a,d). In the C127 cells that expressed EGFP-tagged Spop, we analyzed for subcellular localization of macroH2A1.2 protein using specific antibody. Immunofluorescence staining revealed macroH2A1.2 to be in subcellular speckles (Fig. 4b,e). Ectopically expressed EGFP-tagged Spop shows a similar intranuclear distribution pattern to that of the endogenous macroH2A1.2.

These results indicated that macroH2A1.2 and Spop are present in the form of a complex in vivo.
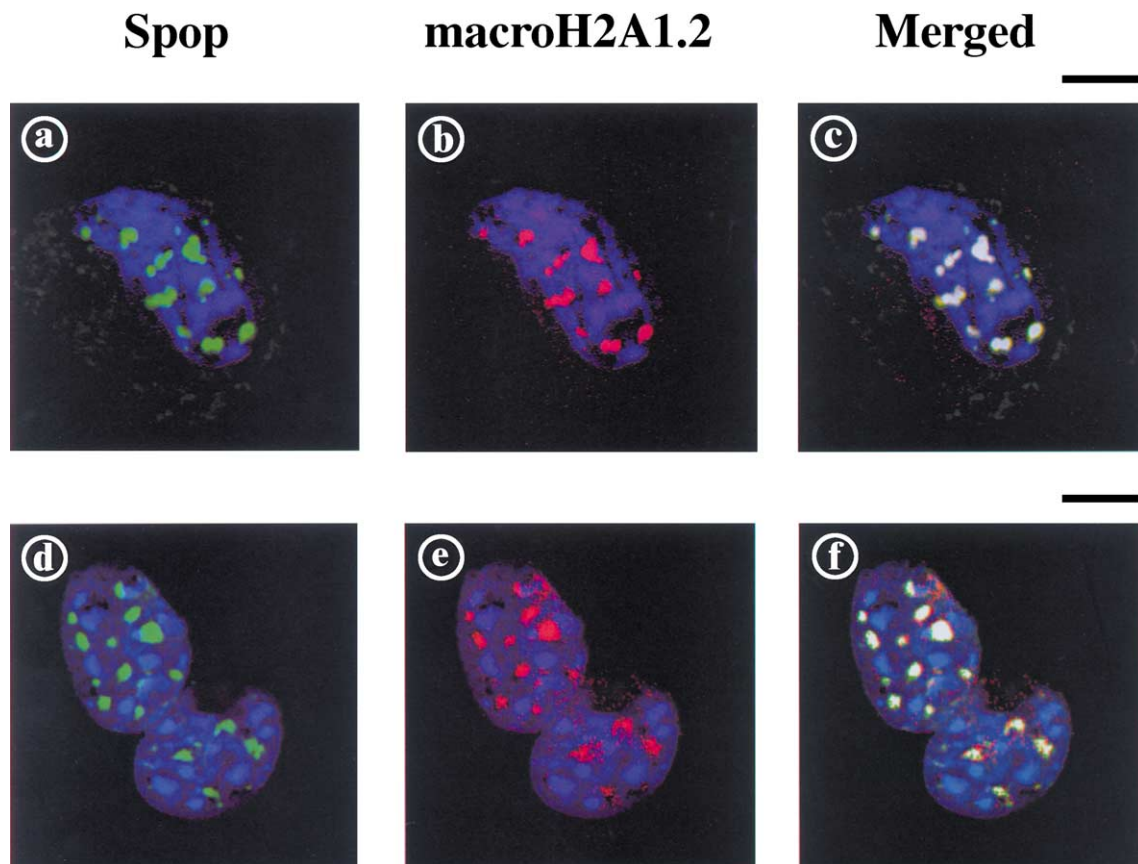

Fig. 4. Co-localization of Spop and macroH2A1.2. C127 cells were transiently transfected with the plasmid expressing the pEGFP–Spop. The cells were then immunostained and analyzed by confocal microscopy. (a,d) EGFP–Spop (green) was detected by the intrinsic fluorescence. (b,e) MacroH2A1.2 (red) was detected by indirect immunofluorescence and visualized by Texas red-conjugated secondary antibody. Panels c and f are merged images; (c) as (a)+(b), (f) as (d)+(e). Nuclear DNA was stained with DAPI (blue). The bar equals 10 μm.

4. Discussions

We found that the macroH2A1.2 nonhistone region interacts with speckled-type POZ (Spop) protein, and the binding domain search demonstrated that the MATH domain of Spop protein, which consists of an N-terminal MATH domain and a C-terminal BTB/POZ domain, interacts with the macroH2A1.2 nonhistone region.

4.1. MATH domain protein

Tumor necrosis factor receptor associated factor (TRAF) proteins [13] and metalloproteinase family Meprins share a conserved domain, called the MATH (meprin and TRAF homology) domain [14], which is referred to as TD (TRAF Domain) by another group [15].

The mammalian proteins containing the MATH domain can be divided into at least three distinctive subfamilies. The first is defined by the TRAF family (TRAF1–6), this is the true TD (TRAF Domain). The second is Meprin, a class of dimeric extracellular metalloproteinases of the astacin family [16], while the third subfamily consists of MUL [17], USP7 [18], and SPOP [19]. *MUL* is the gene that causes the genetic disease called mulibrey nanism, an autosomal recessive disorder that affects several tissues of mesodermal origin. USP7 is a ubiquitin-specific proteinase that binds the Vmw 110 protein of herpes simplex virus 1 (HSV1), and SPOP protein is a nuclear protein recognized by the serum of a sclerodoma patient [13].

The MATH domain (TD) of TRAF family proteins interacts with TNF-receptors and other adaptor proteins and kinases concerned with cell survival, proliferation, and death signaling.

The MATH domains of Meprin family proteins probably play a role in domain–domain interactions that allow limited proteolysis for removal of the prosequence, and also prevent extensive proteolysis of the subunit [16].

The function of the MATH domain derived from MUL and USP7 remains unknown.

Zapata et al. [15] recently reported that the TD (MATH domain) of human SPOP displays weak interaction with TRAF-1 and TRAF-6, revealing that SPOP is not a physical interactor of TRAF family proteins.

The MATH domain of proteins of each subfamily probably interact with various sorts of proteins.

4.2. Spop protein contains BTB/POZ domain at the carboxyl terminal

The BTB (for Broad Complex, Tramtrack and Bric a brac) or POZ (for poxvirus and zinc finger) is an approximately 120-amino acid conserved, hydrophobic domain generally present at the N terminus of proteins [20].

The BTB/POZ domain mediates homomeric and heteromeric protein–protein interactions, targets the protein to distinct nuclear substructures, and is involved in transcriptional repression or chromatin modeling [21,22].

The BTB/POZ domain of BCL6/LAZ3 and PLZF, as well as other POZ domains, may associate with the SMRT–mSin3A–HDAC-1 complex and form a multimeric repressor complex involving histone deacetylation activity [23]. Accordingly, inhibitors of HDAC activity reduce the repressive effect of both LAZ3 and PLZF on their respective cognate sequence, suggesting that they repress transcription, at least in part, by creating a repressive (hypoacetylated) chromatin structure.

At present, we have no evidence that the Spop protein recruits chromatin-modeling components.

4.3. Implications of X chromosome inactivation

Xist RNA plays a central role in X chromosome inactivation and is required for initiation of X inactivation but not for its maintenance. Xist RNA covers the X chromosome "chosen" to be inactivated, and macroH2A1.2 simultaneously concentrates with the inactive X chromosome to form a macrochromatin body (MCB) [24,25]. When Xist RNA ceases to be expressed from the inactivated X chromosome, MCB disappears [4]. It is not known how the macroH2A1.2 protein targets the inactive X chromosome (Xi) and cooperates with Xist RNA in silencing gene expression on Xi.

The histone H2A homologous region of macroH2A1.2 is required for association with the inactive X chromosome [3], and the putative leucin zipper motif of C-terminal of macroH2A1.2 is likely to be a protein–protein interaction motif [8]. Actually, we have shown this putative leucine zipper motif of macroH2A1.2 bound to the Spop protein (see Fig. 4).

On the other hand, it is known that proteins having the BTB/POZ domain can oligomerize among themselves [26,27], and the BTB/POZ domain of Spop proteins binds them to each other (data not shown). Our results seem to suggest that Spop protein oligomerization causes macroH2A1.2 to concentrate on inactive X chromosomes to form of MCB.

Perche et al. [7] reported that the nonhistone region of macroH2A can repress transcription from thymidine kinase promoter in HeLa cells. This effect means that the nonhistone region of the macroH2A1.2 may recruit HDAC complex through the POZ domain of the Spop. The polycomb group gene, eed (embryonic ectoderm development), was recently reported to maintain imprinted X inactivation [28], and this eed protein is known to interact with histone deacetylase (HDAC) [29]. The above two examples indicate that the HDAC complexes may participate in the gene silencing process in X chromosome inactivation. However, the addition of HDAC inhibitor such as TSA or sodium butylate prevented the increase in acetylated histones on inactive X chromosomes [30]. This suggests that other pathways or other mechanisms of gene silencing are present on inactive X chromosomes.

5. Accession numbers

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