Human MutS Homologue MSH4 Physically Interacts with von Hippel-Lindau Tumor Suppressor-binding Protein 1

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ABSTRACT

Increasing evidence indicated that the protein factors involved in DNA mismatch repair (MMR) possess meiotic functions beyond the scope of DNA mismatch correction. The important roles of MMR components in meiotic processes have been highlighted by the recent identification of two additional members of the mammalian MutS homologues, MSH4 and MSH5. Mammalian MSH4 and MSH5 proteins form a heterodimeric complex and play an important role in the meiotic processes. As a step forward to the understanding of the molecular mechanisms underlying the roles of these two mammalian MutS homologues, here we have identified von Hippel-Lindau (VHL) tumor suppressor-binding protein 1 (VBP1) as an interacting protein partner for human MSH4 (hMSH4). In addition, we have characterized a hMSH4 splicing variant (hMSH4sv) encoding a truncated form of hMSH4. The protein encoded by hMSH4sv was unable to interact with hMSH5, but it retained the capacity to interact with VBP1. It is conceivable that hMSH4 and hMSH4sv can carry out different but overlapping functions by differential protein interactions, and, therefore, hMSH4sv might represent a separation-of-function alternative form of the hMSH4 protein. hMSH4 and VBP1 proteins were colococalized in mammalian cells. Three-hybrid analysis suggested that VBP1 could compete with hMSH5 for the binding of hMSH4. Thus, hMSH4 may be involved in diverse cellular processes through interaction with different protein partners, and the levels of VBP1 protein expression in cells could potentially affect the availability of the hMSH4-hMSH5 hetero-complex.

INTRODUCTION

DNA repair mechanisms are involved in both mitotic and meiotic cell divisions, and the integrity of genetic information passed from parental cells to daughter cells is controlled by multiple cellular proteins involved in cell-cycle regulation, DNA replication, DNA repair, and chromosome segregation. Among many DNA repair pathways, DNA MMR1 defines one of the most important molecular mechanisms in maintaining the faithful transmission of genetic information during DNA replication. Mammalian MutS homologues represent important evolutionary conserved components involved in multiple biological functions such as DNA MMR, mitotic and meiotic recombination, and cellular responses to DNA damages (1). The MMR process is initiated with the recognition and binding of the mismatched nucleotides by heterodimeric MutS homologous proteins (2). Mutations in several human MMR genes are linked to the pathogenesis of hereditary nonpolyposis colorectal cancer (HNPCC) and sporadic tumors associated with microsatellite instability (1). Eukaryotic MutS homologous proteins MSH2, MSH3, and MSH6 are proteins known to participate in DNA MMR through the actions of their heterodimeric complexes consisting of either MSH2-MSH3 or MSH2-MSH6, in which the MSH2-MSH6 heterodimer recognizes both single-base mismatches and small loops formed by insertions or deletions in the DNA, whereas the MSH2-MSH3 heterodimer only recognizes small insertions and deletions (3, 4).

Recent evidence demonstrates that eukaryotes contained a separate and functionally related group of MutS homologues including MSH4 and MSH5 (5–10). Diploid Saccharomyces cerevisiae lacking the MSH4 or MSH5 gene display decreased levels of spore viability and reciprocal exchange between homologous chromosomes, along with an increase in nondisjunction of homologous chromosomes at meiosis I (5, 6). Similar to their yeast homologues, mammalian homologous MSH4 and MSH5 proteins are also found to interact and form a heterodimeric structure (9–13). In contrast to MSH5, the expression of human and mouse MSH4 is relatively restricted to meiotic tissues (7, 10). Examination of mouse gene knockout models indicates that Msh4 and Msh5 act in the same pathway during meiosis, and mice carrying the disrupted Msh4 or Msh5 gene display defective chromosome synapsis, resulting in testicular and ovarian degeneration and, therefore, male and female sterility (14–16). In contrast, yeast MSH4 and MSH5 are not essential for the completion of meiotic processes (5, 6), raising the possibility that the mammalian homologous proteins may have evolved to carry out additional cellular functions. It is conceivable that mammalian MSH4-MSH5 heterodimer functions in conjunction with the MLH1-PM252 complex, as supported by the fact that both MLH1 and PMS2 have meiotic functions, and by the recent observation that hMSH4 physically interacts with human MLH1 (17). However, the effect of Msh4 or Msh5 deficiency on the complete disruption of ovarian development is unique among genes known to function in meiotic processes, raising the possibility that mammalian MSH4 and MSH5 could be involved in cellular pathways other than its role in homologous recombination. However, the protein factors that may act together with hMSH4 and hMSH5 in cellular pathways are unknown at present time.

To gain a better understanding of the biological processes associated with hMSH4 in humans, we report here the characterization of a hMSH4sv and the identification of VBP1 as an interacting protein for both hMSH4 and hMSH4sv. VBP1 was first identified as a binding factor for VHL tumor suppressor protein in humans (18). Homologues of VBP1 in other mammals and yeast were highly conserved; VBP1 proteins in human and mouse are 100% identical in amino acid sequences (18–20). Although the precise functions of VBP1 are elusive at present time, it has been shown that VBP1 plays an essential role in promoting the formation and assembly of functional tubulins, suggesting a function in the early steps of microtubule assembly pathway (20–22). Our current data suggest that hMSH4 associates with VBP1 both in vitro and in vivo.

Received 8/6/02; accepted 12/13/02.

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1 Supported in part by an American Cancer Society Institutional Research grant.

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3 The abbreviations used are: MMR, mismatch repair; hMSH4, human MSH4; hMSH4sv, hMSH4 splicing variant; VHL, von Hippel-Lindau; VBP1, VHL tumor suppressor-binding protein 1; IPTG, isopropyl-β-D-thiogalactopyranoside; BID, binding domain; AD, activating domain; ORF, open reading frame; UTR, untranslated region; GST, glutathione-S-transferase; SD, synthetic dropout; UAS, upstream activating sequence; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HA, hemagglutinin; RAGE, rapid amplification of genomic DNA ends; BAC, bacterial artificial chromosome; ECL, enhanced chemiluminescence; GFP, green fluorescent protein.
MATERIALS AND METHODS

Yeast Two-Hybrid Library Screening and Two- or Three-Hybrid Analysis. Human MutS homologue hMSH4 was used as a bait protein in the yeast two-hybrid screening of a human ovary cDNA library (Clontech), in which we have identified VBPI as an interacting protein partner for hMSH4. Specifically, two-hybrid vector pAS2–1 containing full-length hMSH4 in frame with GAL4-BD was used to perform two-hybrid screening of a human ovary cDNA library in strain Y187. We have screened approximately 3.5 × 10^6 clones of the human ovary cDNA library on SD/-Ade-Leu-His-Trp medium for the selection of positive protein-protein interactions by transcription activation of ADE2 and HIS3 reporter genes. All of the subsequent yeast two-hybrid analyses were carried out by the use of the two-hybrid vectors, pAS2–1 or pGBK7 and pAct2 or pGADT7 (Matchmaker Two-Hybrid System; Clontech), as well as S. cerevisiae strains Y187, Y190, or AH109. Yeast transformants harboring both DNA-BD and activation domain constructs were selected on SD/-Leu-Trp medium. Positive protein-protein interactions were ascertained by the transcription activation of highly inducible GAL1 UAS driving lacZ gene in the reporter host strains Y187 and Y190, as well as histidine prototrophy performed with Y190 or AH109 double transformants on SD/-Leu-Trp-His medium. β-galactosidase activities were qualitatively monitored by the blue color development with X-gal filter assays.

The complex protein interactions among hMSH4, hMSH5, and VBPI were analyzed with a three-hybrid approach (23), which was based on the expression of an additional protein in its “native” form together with the expression of a DNA-BD fusion protein and an activation domain fusion protein within the same yeast cells. To generate constructs for the three-hybrid analysis, we have cloned hMSH4, VBPI, and hMSH5 cDNAs into pBridge and pAct2 vectors (Clontech). Briefly, each pBridge construct is designed to express two of the three concerned genes as a BD-fusion and a HA-tagged protein. The pBridge vector contains two cloning sites; the first cloning site is for the generation of cloned hMSH4, VBPI, and hMSH5 cDNAs into pBridge and pAct2 vectors (Clontech). The complex protein interactions among hMSH4, hMSH5, and VBPI were ascertained by the transcription activation of a BD-fusion and a HA-tagged protein. The pBridge vector contains two cloning sites; the first cloning site is for the generation of cloned hMSH4, VBPI, and hMSH5 cDNAs into pBridge and pAct2 vectors (Clontech).

A. hMSH4 Gene Organization

Fig. 1. Human hMSH4 gene structure and multiple tissue cDNA panel-based analyses of human hMSH4 and hMSH4sv expression. A, hMSH4 gene organization. Black rectangles, portions of exons that encode ORF sequences; open rectangles, 5' and 3' UTRs. The hMSH4 exon 19 that is absent from hMSH4sv. B, analysis of human hMSH4 and hMSH4sv expression. PCR amplifications were performed with hMSH4 cDNA-specific primers, forward F2273 and reverse R2685 located on hMSH4 gene exons 17 and 20 (arrows in A), and designed to amplify 413-bp and 324-bp products representing hMSH4 and hMSH4sv, respectively.
(Schleicher and Schuell, Keene, NH) at 15 V for 2.5 h with a semidry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). Immunoreactive proteins were detected with the ECL Western blotting system (Amersham Pharmacia Biotech). Affinity-purified rabbit α-hMSH4 polyclonal antibody was generated against a synthetic peptide corresponding to hMSH4 amino acid residues 920–936 (Y-K-E-D-F-P-R-T-E-Q-V-P-E-K-T-E-E), with the addition of a cysteine residue at the NH2 terminus of the peptide to mediate its coupling to keyhole limpet hemocyanin (Zymed Laboratories Inc., South San Francisco, CA). Other antibodies used in this study include α-T7 tag monoclonal (Novagen, Madison, WI), α-HA monoclonal 12CA5 (Boehringer Mannheim), α-Gal4 AD, and α-Gal4 BD monoclonal antibodies (Clontech).

**In Vitro Binding Assay.** In vitro binding assay was used to validate the interaction between hMSH4 and VBP1. To generate recombinant proteins fused to either GST or His6-T7 tag, the hMSH4 and VBP1 coding sequences were cloned in-frame into pGEX-6p (Pharmacia, Piscataway, NJ) and pTct (Invitrogen, San Diego, CA) vectors using a PCR-based approach. Native *Pyrococcus furiosus* DNA polymerase (Stratagene, La Jolla, CA) was used in all of the PCR amplifications. Fusion proteins were produced in either *Escherichia coli* BL21-Gold or BL21(DE3)-RIL strains (Stratagene). Overnight induction of protein expression was carried out with 0.4 mM IPTG at 16°C. In vitro binding assay was performed essentially as described in detail elsewhere (24). Briefly, the GST-VBP1 and GST control were purified with glutathion Sepharose 4B (Amersham Pharmacia Biotech). Immobilized GST or GST-VBP1 on glutathion Sepharose 4B was then used to capture the second recombinant protein His6-hMSH4. Similarly, the TALON Metal Affinity Resin (Clontech) vectors were used to immobilize recombinant His6-VBP1 and a His6-tag control protein, His6-GFP and then were used to capture recombinant protein GST-hMSH4. Bound proteins were eluted with SDS sample buffer and subjected to Western blot analysis for the presence of hMSH4 fusion proteins.

**Immunoprecipitation.** Communoprecipitation analysis of hMSH4 and VBP1 interaction was carried out by the use of bacterial expressed recombinant fusion proteins. pGEX-6p and pET-28a (Novagen) vectors were used to generate expression constructs for fusion proteins GST-hMSH4 and His6-VBP1 containing the T7 tag epitope. Coexpression of recombinant proteins from pGEX-6p- and pET-28a-based constructs was carried out in *E. coli* BL21(DE3)-RIL with the addition of 0.4 mM IPTG to induce protein expression at 16°C overnight. Soluble fractions of whole-cell lysate were prepared in PBS containing 150 mM NaCl, 1 mM MgCl2, 10% glycerol, and 1 mM DTT. Cell lysates were then incubated overnight with 5 μg of affinity-purified α-hMSH4 polyclonal or 2.5 μg of monoclonal α-T7 tag antibody at 4°C. Immunoprecipitates were captured with 75 μl of 50% slurry of BSA-saturated Protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Affinity-purified rabbit anti-hMSH4 antibody at 4°C. Immunoprecipitates were captured with 75 μl of 50% slurry of BSA-saturated Protein A/G PLUS-Agarose (Santa Cruz Biotechnology).

**Colocalization of hMSH4 and VBP1 in Mammalian Cells.** Subcellular colocalization of hMSH4 and VBP1 was carried out by the use of fluorescent fusion proteins. To generate GFP-hMSH4 and VBP1-DsRed2 proteins, hMSH4 and VBP1 cDNAs were subcloned into pEGFP and pDsRed2 (Clontech) vectors, respectively. The resulting expression constructs were transfected into 293T cells (25, 26), and transfected cells were maintained in DMEM supplemented with 10% newborn calf serum at 37°C and 5% CO2 for 48 h. Confocal images of subcellular distributions of fluorescent hMSH4 and VBP1 fusion proteins were acquired by the use of Nikon Eclipse TE300 hooked to Bio-Rad MRC 1024 laser confocal microscope with LaserSharp 3.0 software (Bio-Rad Laboratories).

**RESULTS**

As a step toward the identification of hMSH4 interacting protein partners, we have isolated hMSH4 ORF sequence by PCR from a human testis cDNA preparation (9). Sequence analysis of cloned hMSH4 ORFs indicated that, in addition to the full-length hMSH4 ORF, we have also identified a 89-bp between nucleotide positions 2530 and 2620 corresponding to the entire exon 19. This alternative splicing resulted in a frameshift of 7 amino acids followed by a stop codon at nucleotides 2640–2642 in exon 20 (Fig. 1). The 850-amino acid-truncated protein encoded by hMSH4sv ORF contained most of the highly conserved sequence motifs of MutS homologues except for the helix-turn-helix motif located within the COOH terminus. hMSH4sv transcripts were expressed predominantly in human testis with an estimated expression level reaching ~50% of the full-length transcript (Fig. 1). Very weak amplification signals for both hMSH4 and hMSH4sv were also observed from human brain, pancreas, colon, and thymus (Fig. 1). These amplification products could not have resulted from genomic DNA contamination because the two primers were located on hMSH4 coding exons 17 and 20 and designed to amplify 413-bp and 324-bp products representing hMSH4 and hMSH4sv transcripts.

To identify protein partners associated with hMSH4, full-length hMSH4 ORF was cloned into a Gal4 two-hybrid vector and was used to screen a human ovary cDNA library. The hMSH4 protein bait identified five individual clones, including four distinct ones, containing cDNA sequences with various lengths but all of them encoded VBP1 protein. The longest VBP1 cDNA encoding full-length VBP1 protein was used for all of the subsequent analyses. Yeast two-hybrid analyses demonstrated that hMSH4 specifically interacted with VBP1 (Table 1). Considerable levels of β-galactosidase activities were obtained when hMSH4-BD and VBP1-AD, or VBP1-BD and hMSH4-AD were coexpressed in Y187 and Y190, suggesting that hMSH4 associates with VBP1 (Table 1). On the contrary, there was no observed interaction between hMSH5 and VBP1 (Table 1). Interestingly, the truncated protein encoded by hMSH4sv also interacted with VBP1 with strength equivalent to that between hMSH4 and VBP1, suggesting that the protein encoded by the splicing variant could be functional. However, the truncated hMSH4sv protein could not interact with hMSH5, indicating that the helix-turn-helix motif located at the COOH terminus of hMSH4 is critical for interaction with hMSH5 (Table 1). A separate reporter gene HIS3 in strain Y190 or AH109 was also used to validate the observed protein interactions. As shown in Table 1, histidine prototrophy phenotypes were ascertained from Y190 or AH109 double transformants harboring the same pairs of two-hybrid constructs that could lead to LacZ gene activation. The observed protein interactions were further substantiated when we

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**Table 1** Two-hybrid analysis of hMSH4 and VBP1 interactions

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failed to observe LacZ and HIS3 gene activation in yeast double transformants expressing only one of the two testing proteins in either BD or AD fusion form (Table 1).

An in vitro binding assay was then performed to investigate the interaction between hMSH4 and VBP1. To generate GST-fusion and His6-T7 tagged proteins, the hMSH4 and VBP1 coding sequences were cloned in-frame into pGEX-6p and pTrcHis vectors and introduced into BL21(DE3)-RIL cells for the generation of recombinant proteins. The GST-VBP1 and GST control proteins were purified from soluble fraction and immobilized onto glutathione-Sepharose beads. Immobilized GST-VBP1 or GST proteins on glutathione-Sepharose beads were then incubated with bacterial lysates containing recombinant His6-T7-hMSH4 fusion protein. Proteins that retained on glutathion-Sepharose beads were eluted in sample buffer and separated by SDS-PAGE, which was followed by transferring onto nitrocellulose membranes and immunoblotting with affinity-purified α-hMSH4 antibody. Apparently, GST-VBP1 fusion protein, but not GST alone, could result in the retention of hMSH4 protein (Fig. 2A). Similarly, the immobilized recombinant His6-T7-VBP1 protein on TALON metal affinity resin could capture GST-hMSH4 protein, whereas immobilized control fusion protein His6-GFP did not display the same property (Fig. 2B). Consistent with the results of the two-hybrid analysis, the in vitro binding assay demonstrated that recombinant VBP1 protein specifically interact with recombinant hMSH4.

To address whether this interaction could occur in solution between hMSH4 and VBP1, immunoprecipitation experiments were performed with the soluble fraction of the bacterial lysates containing both hMSH4 and VBP1 fusion proteins. Recombinant GST-hMSH4 and His6-T7-VBP1 fusion proteins were coexpressed in BL21(DE3)-RIL host cells. Soluble fractions of cell lysates were prepared from cells expressing both fusion proteins and cells expressing either of the two, followed by incubation with α-hMSH4 or monoclonal α-T7 tag antibodies. Immunoprecipitants were analyzed by immunoblotting with α-hMSH4 or monoclonal α-T7 tag antibodies. The results of these experiments demonstrated that the affinity-purified polyclonal α-hMSH4 and monoclonal α-T7 tag antibodies were capable of immunoprecipitating GST-hMSH4 and His6-T7-VBP1 fusion proteins, respectively (Fig. 2C, Lanes 1 and 5). Monoclonal α-T7 tag antibody against VBP1 fusion protein could precipitate hMSH4 only when VBP1 was present (Fig. 2C, Lanes 2 and 4). Furthermore, immunoprecipitation of VBP1 with affinity-purified α-hMSH4 antibody was entirely dependent on the expression of hMSH4 protein (Fig. 2C, Lanes 6 and 7); His6-T7-VBP1 was detected only when lysate containing both hMSH4 and VBP1 was used but not with the control lysate expressing VBP1 and GST (Fig. 2C, Lanes 6 and 7). Taken together, these results suggest that hMSH4 specifically interacts with VBP1 in solution.

We next set out to identify hMSH4 domains responsible for interaction with VBP1 using the two-hybrid approach. As shown in Fig. 3, a series of deletion hMSH4 mutants were generated in GAL4-based BD or AD vectors and were used to perform two-hybrid analyses. Positive protein interactions were ascertained by the transcription activation of highly inducible GAL1 UAS driving lacZ gene in the reporter strains and were determined qualitatively with the X-gal filter assay. The results of these analyses indicated that both NH2 and COOH termini of hMSH4 were not required for interaction with VBP1 (Fig. 3). In particular, hMSH4 deletion mutant, lacking the first 147 amino acid residues, maintained the ability to interact with VBP1; however, any further deletion from the NH2 terminal abolished that interaction (Fig. 3). This observation indicated that the first 147 amino acid residues of hMSH4 were not involved in mediating protein interaction with VBP1, coupling the fact that this portion of the amino acid sequence is highly divergent between human and mouse MSH4 (10). Although hMSH4 amino acid residues 844–936 containing the helix-turn-helix motif were not required for interaction with VBP1, as suggested by the observed interaction between hMSH4sv and VBP1, further deletion of the region connecting the putative nucleotide-binding motif and helix-turn-helix motif abolished the interaction (Fig. 3). In addition, all of the other truncated mutations, containing different lengths of hMSH4 peptide sequences, would not interact with VBP1 (Fig. 3). Therefore, it is conceivable that most of the hMSH4 peptide sequence is required for mediating protein interac-
in vivo could occur interacting region is composed of multiple noncontinuous regions of interactions with VBP1. Alternatively, it is also possible that the VBP1-interacting region is composed of multiple noncontinuous regions of hMSH4 protein.

To demonstrate that the association of hMSH4 and VBP1 proteins could occur in vivo, we have attempted to determine whether hMSH4 colocalizes with VBP1 in cultured mammalian cells. To this end, MSC-1 cells were transfected with either an individual construct or a mixture of both expression constructs encoding GFP-tagged hMSH4 or DsRed2-tagged VBP1 proteins. As shown in Fig. 4, VBP1 was predominately localized in the cytoplasm, particularly displayed as perinuclear foci. The observed VBP1 subcellular distribution pattern was nearly identical, as reported previously (18). Although hMSH4 distributed diffusely in the cytoplasm without concomitant expression of VBP1 (Fig. 4A), it appeared that hMSH4 fusion protein could redistribute and accumulate prominently within VBP1 foci in cells expressing both proteins (Fig. 4C). Together, these data suggested that hMSH4 and VBP1 proteins resided predominantly in the cytoplasm, and VBP1 could trigger the formation of hMSH4-VBP1 foci.

It is known that mammalian MSH4 forms heterocomplex with MSH5 and mice lacking either functional Msh4 or Msh5 displayed similar meiotic phenotypes, suggesting that these genes might function in the same biological pathway (9–13). Therefore, the observation that hMSH4 could interact with VBP1 raised interesting questions regarding whether VBP1 would compete with hMSH5 for the binding of hMSH4. We next attempted to investigate the interplay of hMSH4, VBP1, and hMSH5 proteins with regard to the potential formation of protein complex. We have adapted the three-hybrid approach to determine whether there was a cooperative or competitive interaction among these three proteins. As indicated in Fig. 5A, the activation of both HIS3 and ADE2 reporter genes, as determined by the growth of yeast transformants on SD/-Ade-Leu-His-Trp medium, were obtained only when BD-hMSH4 and AD-hMSH5 or BD-hMSH4 and AD-VBP1 were coexpressed in host strain AH109 (yeast transformants no. 13 and no. 15). However, the activation of these reporter genes was almost completely inhibited when HA-VBP1 or HA-hMSH5 was coexpressed in the corresponding yeast transformants (no. 14 and no. 16). These observations suggested that VBP1 might compete with hMSH5 for the binding of hMSH4. This view was also supported by the lack of reporter gene activation in yeast transformant no. 18 (coexpressing BD-hMSH5, AD-VBP1, and HA-hMSH4) and transformant no. 20 (coexpressing BD-VBP1, AD-hMSH5, and HA-hMSH4), suggesting that hMSH4 could not interact with VBP1 and hMSH5 concomitantly. Similar experiments performed with a separate reporter strain Y187 provided identical results (data not shown). The expression of relevant fusion proteins in the concerned AH109 transformants was validated with Western blot analysis, so that the failure in reporter gene activation was not attributable to the absence of protein expression (Fig. 5B). These observations were further supported when we failed to observe reporter gene activations in AH109 double transformants coexpressing only one of the three testing proteins in either AD or BD fusion form (Fig. 5A, transformants no. 1 to no. 6). Furthermore, AH109 transformants coexpressing any combination of two proteins in BD-fusion and native forms could not grow on SD/-Ade-Leu-His-Trp medium (Fig. 5A, transformants no. 7 to no. 12). In contrast, all of the AH109 double transformants grew efficiently on SD/-Leu-Trp medium (Fig. 5A; bottom panel). These results indicated that hMSH4-hMSH5 heterodimer could be disrupted by the overexpression of VBP1 protein.

**DISCUSSION**

In the present study, we aimed to identify human hMSH4-associating protein partners and to understand their potential effects on the formation of hMSH4 and hMSH5 heterocomplex. With the full-length hMSH4 as bait, we have identified VBP1 as a putative protein-binding partner for hMSH4. The *in vitro* binding assay demonstrated a direct interaction between hMSH4 and VBP1 proteins, which was further confirmed by immunoprecipitation experiments (Fig. 2). Moreover, hMSH4 associated with VBP1 and formed cytoplasmic foci in MSC-1 cells (Fig. 4). We have also identified and characterized a hMSH4sv that encoded a truncated protein lacking the conserved COOH terminal helix-turn-helix motif. Although the truncate hMSH4sv protein could not interact with hMSH5, it interacted with VBP1 at approximately equivalent strength as the full-length protein (Table 1), suggesting that hMSH4sv might represent a separation-of-function alternative. This observation leads to the possibility that hMSH4sv protein might be functional and, therefore, involved in the same pathway of VBP1. In addition, the inability of hMSH4sv to interact with hMSH5 indicates that the conserved helix-turn-helix motif of hMSH4 is critical for protein interaction with hMSH5. It is conceivable that the human hMSH4 might encode at least two functional distinct proteins; the full-length protein could interact with both hMSH5 and VBP1, whereas the alternative form interacts only with VBP1. Both hMSH4 and hMSH4sv transcripts were expressed predominantly in human tissues.
testis; the level of hMSH4sv expression was \(~50\%\) of that of the hMSH4 (Fig. 1)

Homologues of the human VBP1 protein were highly conserved in mammals and yeast (18–20). It has been shown that VBP1 played an essential role in promoting the formation and assembly of functional \(\alpha\)-, \(\beta\)-, and \(\gamma\)-tubulin; thus, it functions in the early steps of the microtubule assembly pathway (20–22). It is known that microtubules play an important role in numerous cellular processes including chromosome segregation in mitosis and meiosis. Thus, the observed interaction between VBP1 and hMSH4 (or hMSH4sv) proteins suggested that hMSH4 might possess multiple functions during the meiotic process; besides its role in meiotic recombination, hMSH4 might be directly or indirectly involved in the manipulation of microtubule assembly to maintain proper chromosome positioning during meiotic processes. It has been shown that disruption of the yeast VBP1 homologous gene resulted in the generation of malfunctioning spores and, therefore, leads to germination defects (27). Yeast VBP1 homologue was not essential for vegetative growth; however, deletion mutants lacking the functional gene displayed a slow growth phenotype (27). Previous studies performed with mammalian MSH4 and MSH5 indicated that these two proteins could form a heterocomplex, and targeted mutation of either Msh4 or Msh5 in mice caused pachytene arrest because of disrupted chromosome pairing and, therefore, female and male sterility (9, 10, 12–16). Interestingly, results of our current study have suggested that VBP1 could compete with hMSH5 for the binding of hMSH4, raising the possibility that hMSH4 might play multiple functional roles through interactions with different protein partners. It appeared, however, that the existence of hMSH4sv protein might represent a mechanism to alleviate the competitive tension between VBP1 and hMSH5 to form protein complex with hMSH4. Of course, the specific functions associated with these two protein complexes and the question as to when they become critical during the meiotic process remain to be clarified. Another interesting question is whether the expression of hMSH4 and hMSH4sv proteins is subject to coordinate regulation. Although the stoichiometry of the functional protein complex consisting of hMSH4 and VBP1 has not yet been directly evaluated, several lines of evidence indicate that the formation of hMSH4-VBP1 multimeric functional complex, such as a tetramer, is theoretically possible. It has been shown that both yeast and human MSH4 was able to form homo-oligomer structures (9, 11), and the human VBP1 protein could mediate strong homotypic interactions in a yeast two-hybrid analysis (data not shown).

MSH4 and MSH5 were not required for the completion of meiosis in both yeast and Caenorhabditis elegans (5, 6, 28, 29); conversely, the successful completion of meiosis in mice required functional Msh4 and Msh5 genes (14–16). In addition to meiotic defects, inactivation of either Msh4 or Msh5 in mice resulted in testicular and ovarian degeneration; inactivation of these two genes had more severe effects on ovary developments because complete ovarian dysgenesis in Msh4- and Msh5-deficient mice by adulthood was observed apparently as a result of massive apoptosis (14–16). These findings are particularly important in light of the fact that no other genes known to be involved in meiotic processes associate with this profound phenotype; coupled with the fact that yeast VBP1 mutants display a growth defect (27), the current data are consistent with the view that the hMSH4-VBP1 interaction might be involved in cellular processes critical for ovarian development. To this end, it would be of interest to know whether hMSH4-VBP1 and hMSH4sv-VBP1 complexes would possess similar functional properties and whether there is any spatial and temporal control for the formation of different heterocomplexes among these three proteins.

Fig. 4. Subcellular colocalization of hMSH4 and VBP1 in MSC-1 cells. Confocal microscopic analysis of hMSH4 and VBP1 localization was carried out with GFP or DsRed2 fluorescent fusion proteins, respectively. A, MSC-1 cells were transfected with hMSH4/pEGFP plasmid DNA. B, MSC-1 cells were transfected with VBP1/pDsRed2 construct. C, MSC-1 cells were cotransfected with hMSH4/pEGFP and VBP1/pDsRed2 plasmids. Confocal images were acquired at different magnifications for cells expressing individual proteins (A, B) and cells coexpressing both fusion proteins (C).
Our present study also indicated that hMSH5 was predominately presented in the cytoplasm with subcellular localization patterns that were similar to those of hMSH4 (data not shown). Hence, the translocation of these proteins into the nucleus must be regulated by a yet-to-be-identified mechanism (14–16). One important aspect in the functional analysis of these protein complexes is to determine whether they have functions beyond the scope of meiosis. It is known that mammalian VBP1 is ubiquitously expressed in adult tissues (18, 19, 30). In contrast to the meiotic-restricted expression pattern of mammalian MSH4, transcripts, and in some cases proteins of human hMSH5, have been identified in almost all of the tissues examined (Refs. 8, 12, 13; data not shown). Consistent with a previous report that yeast MSH5 is also involved in mitotic processes (31), these observations suggest that hMSH5 might play functional roles in human nonmeiotic tissues either in the absence or in the presence of hMSH4. In summary, our present study suggests that VBP1 may act as a protein partner for both hMSH4 and hMSH4sv during meiotic processes; it is plausible that the cellular effect of hMSH4-VBP1 interaction is on the manipulation of microtubule assembly during meiotic chromosome segregation and, therefore, plays a role in the maintenance of chromosomal stability. Meiotic nondisjunction is the major cause for aneuploidy, which leads to physical abnormalities and mental retardation in humans (32). One may expect that disruption of protein interactions because of mutations of these protein factors can cause meiotic defects in humans.

ACKNOWLEDGMENTS

We thank Dr. Norman Doggett, Dr. Cliff Han, Dr. Hong Cao, Tai-Hsien Lee, Robert Adair, and Dr. DongKee Jeong for their helpful advice and assistance.

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Fig. 5. Analysis of complex protein interactions among hMSH4, VBP1, and hMSH5. All of the relevant constructs used in the three-hybrid analysis were generated with pBridge and pACT2 vectors, and relevant constructs were transformed into S. cerevisiae reporter strain AH109. Positive protein-protein interactions were ascertained by simultaneous transcription activation of inducible GAL1 UAS driving HIS3 reporter gene and GAL2 UAS driving ADE2 reporter gene in the reporter host strain AH109, which displayed adenine (Ade) and histidine (His) prototrophy phenotypes as measured on SD/-Ade-Leu-His-Trp medium. A, 20 AH109 double transformants, expressing one, two, or three fusion proteins representing different combinations of hMSH4, VBP1, and hMSH5 in BD, AD, or HA fusion forms, were spotted at various dilutions on selection medium SD/-Ade-Leu-His-Trp (top panel), and with single dilution on SD/-Leu-Trp (bottom panel). AH109 transformants no. 1 to no. 12, negative controls for the analysis. B, validation of expression of relevant fusion proteins in representative AH109 double transformants by Western blot analysis performed with α-hMSH4, α-HA, α-Gal4 AD, and α-Gal4 BD antibodies. kDa, molecular weight (Mr) in thousands.

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