

Nuclear localization of human DNA mismatch repair protein exonuclease 1 (hEXO1)

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ABSTRACT

Human exonuclease 1 (hEXO1) is implicated in DNA mismatch repair (MMR) and mutations in hEXO1 may be associated with hereditary nonpolyposis colorectal cancer (HNPCC). Since the subcellular localization of MMR proteins is essential for proper MMR function, we characterized possible nuclear localization signals (NLSs) in hEXO1. Using fluorescent fusion proteins, we show that the sequence ⁴¹⁸KRPR⁴²¹, which exhibit strong homology to other monopartite NLS sequences, is responsible for correct nuclear localization of hEXO1. This NLS sequence is located in a region that is also required for hEXO1 interaction with hMLH1 and we show that defective nuclear localization of hEXO1 mutant proteins could be rescued by hMLH1 or hMSH2. Both hEXO1 and hMLH1 form complexes with the nuclear import factors importin $\beta/\alpha1,3,7$ whereas hMSH2 specifically recognizes importin $\beta/\alpha3$. Taken together, we infer that hEXO1, hMLH1 and hMSH2 form complexes and are imported to the nucleus together, and that redundant NLS import signals in the proteins may safeguard nuclear import and thereby MMR activity.

INTRODUCTION

Although initial recognition steps of DNA mismatch repair (MMR), carried out by the hMSH2-hMSH6 (hMutS α), hMSH2-hMSH3 (hMutS β) and hMLH1-hPMS2 (hMutL α) complexes, have been relatively well studied, the nature of the downstream steps is less characterized (1). One of these downstream steps is the exonucleolytic removal of the DNA strand containing the mispaired nucleotide by one or more exonucleases.

So far only a single exonuclease—human exonuclease 1 (hEXO1)—has been identified in humans (2). Due to endonucleolytic activity of hMLH1 and 5'-exonuclease activity of hEXO1 bidirectional MMR can occur in the presence of a single exonuclease (3–5). The coordinated activity between proteins in the MMR pathway is supported by the findings that specific interactions exist between hEXO1 and hMutS α , hMutS β as well as hMutL α ; and that these interactions control enzymatic activities (3–13). However, whether they are also important for subcellular localization of the MMR proteins remains to be elucidated.

Hereditary nonpolyposis colorectal cancer (HNPCC) patients are characterized by defects in the MMR system. In the majority of the patients, mutations are located in one of the four MMR genes participating in the MutLS complexes—*hMLH1*, *hMSH2*, *hPMS2*, and *hMSH6*—with *hMLH1* and *hMSH2* accounting for the majority of the disease-causing mutations (14). In the so-called atypical HNPCC families that fulfill some but not all of the Amsterdam criteria only a small proportion of individuals exhibit mutations in any of the four MMR genes (14). Germline variants of *hEXO1* have been detected in some patients with atypical HNPCC (15). Moreover, *Exo1* mutant mice are characterized by reduced survival, increased susceptibility towards development of lymphomas and increased mutation rates (16,17). The phenotype of *Exo1* mutant cells is comparable to *MSH6* mutant cells and mutations in the human homolog of this gene have been identified at a high incidence in patients with atypical HNPCC (18–20). It is, therefore, possible that a similar pathology may explain the relation between *hEXO1* mutations and HNPCC development, resulting in late onset of disease, deviating tumor spectrum and/or incomplete penetrance. Polymorphisms in the *hEXO1* gene may also have relevance for the development of sporadic cancers. Destabilization of MMR complex formation and the

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resulting onset of cancer will depend on the combination as well as the nature of *hEXO1* and other MMR gene variants (2).

Two splice variants *hEXO1a* (*HEX1*) and *hEXO1b* have been identified and the transcripts give rise to proteins with predicted molecular weights of 89 and 94 kDa, respectively. For the *hEXO1* protein to participate in MMR it needs to enter the nucleus. Molecules smaller than ~60 kDa can passively diffuse through the nuclear pore complexes (NPCs) into the nucleus, whereas import of larger molecules requires specific nuclear localization signals (NLSs) (21). The classical nuclear translocation pathway is mediated by the importin α/β protein complex. The initial cytoplasmic event is the binding of karyophilic protein, via its NLS, to importin α subunit of the heterodimeric receptor. The importin β subunit mediates docking to the NPC followed by translocation into the nucleus by association of importin β with Ran that triggers dissociation (22). Presently, no definite NLS consensus sequence has been determined, although NLS sequences are frequently composed of basic amino acids and may be classified as either mono- or bipartite (23). It has recently been demonstrated that complex formation between MMR proteins is crucial for nuclear import of some of these proteins (24–26) but it is not clear if complex formation between *hEXO1* and *hMSH2/hMLH1* influences the subcellular localization of the proteins involved. The *hMLH1*, *hPMS2*, *hPMS1*, *hMSH3* and *hMSH6* proteins but not *hMSH2* contain potential NLS sequences and some of them also contain nuclear export sequences (NESs), which enable them to shuttle between nucleus and cytoplasm (24,25,27).

The importance of correct subcellular translocation of MMR proteins is highlighted by the fact that defects in nucleocytoplasmic import of repair protein could confer a MMR-deficient phenotype. Along these lines, a significant number of HNPCC mutant proteins show no apparent biochemical defects but cells containing these proteins are nevertheless MMR deficient (14). One explanation for this phenotype could be that these HNPCC mutant proteins show imperfect subcellular location. In order to investigate the mechanism underlying nucleocytoplasmic import of MMR proteins, we characterized possible NLS targeting signals in *hEXO1*. Using fluorescent fusion proteins, we show that the sequence ⁴¹⁸KRPR⁴²¹ is responsible for correct nuclear localization of *hEXO1*.

So far, only one importin β isoform has been identified whereas six human importin α s (importin α 1,3,4,5,6,7) have been described (28). These importin α s seem to be expressed ubiquitously with little tissue-specific variation. They can be grouped into three subfamilies based on sequence homology where importin α 1 belongs to subfamily 1, importin α 3 to subfamily 2 and importin α 7 to subfamily 3. It has been shown that the individual importin α s are able to import the same target proteins (28) and that two competing substrates can change the import capacity of importin α s (29). Therefore, we found it important to identify the individual importin α s that are responsible for nuclear import of the MMR proteins *hMSH2*, *hMLH1* and *hEXO1*. We show that both *hEXO1* and *hMLH1* are able to form complex with

importin α 1,3,7 whereas *hMSH2* specifically recognizes importin α 3. Examination of the *hEXO1* NLS mutant proteins showed that these mutant proteins were able to form complex with importin α 3 despite its cytoplasmic localization and that defective nuclear localization of the NLS mutant proteins could be alleviated by co-expression with either *hMLH1* or *hMSH2*. These results suggest a complex and strictly controlled mechanism for translocation of MMR proteins from cytoplasm to the nucleus. Furthermore, our results suggest a possible mechanism for tissue- or cell-type-specific discrimination of nuclear translocation of MMR proteins.

MATERIALS AND METHODS

Plasmids

Plasmid pLJR115 expressing an NH₂-terminal tagged YFP-*hEXO1b* fusion protein (10) was used as a template to construct a series of YFP-*hEXO1* mutant proteins. The *hEXO1b* allele contained in pLJR115 has previously been described in (7,9,10,12,13). This allele differs from other frequently used *hEXO1b* alleles. The *hEXO1b* protein used in our studies contains proline at position 757 instead of leucine (P757L) otherwise the amino acid sequence of *hEXO1b* is identical to the one expressed from *hEXO1b* (Genbank: AAD13754). Mutations in *hEXO1b* were introduced using QuikChange site-directed mutagenesis according to the manufacturer's guidelines (Stratagene, La Jolla, CA). The DNA sequences of the entire coding region of *hEXO1b* in the resulting constructs were verified by DNA sequencing (ABI-Prism[®] BigDye[™] Terminator Cycle sequencing, Applied Biosystems). Oligonucleotide primers (Supplementary data Table 1) used for site-directed mutagenesis were purchased from Invitrogen. For *in vitro* transcription translation (IVTT) of *hEXO1* mutant protein K418A, plasmid pLJR129 (YFP-*hEXO1*(K418A)) was digested with BamHI and Sall and the fragment containing *hEXO1*(K418A) cloned into the BamHI and XhoI site of pcDNA3.1A(-) (Invitrogen). For IVTT of *hEXO1* wild-type protein, pLJR115 (YFP-*hEXO1*) was digested with BamHI and Sall and the fragment containing *hEXO1b* cloned into the BamHI and XhoI site of pcDNA3.1A(-) (Invitrogen). Other plasmids pcDNA3-*hMLH1*, pcDNA3.1-*hMSH2*, pCITE-*hMSH2*, pCITE-*hMSH2*(G674R), pCITE-*hMSH2* (C697F) and pCITE-*hMSH2*(P622L) used for IVTT have been described elsewhere (10,13; A. Lützen *et al.*, submitted for publication). The plasmids containing the importin α 1, α 3 and α 7 was a gift from Dr Matthias Köhler (29). Importin α 1 and α 3 were cloned into pQE60 vector and importin α 7 into pQE70. All importin α constructs encode COOH-terminal His-tagged fusion proteins. The plasmid containing the murine GST-tagged importin β (pGEX HA-PTAC97) was a gift from Dr Toshihiro Sekimoto (30).

The coding region of *HEX1* was amplified with the PCR Elongase system (Invitrogen) using primers containing additional restriction enzyme sites, primer pair (5'-TAT GTC GAC ATG GGG ATA CAG GGA TTG-3') and (5'-GCA GGA TCC TCA GAA TTT TTT

AAA TCC-3') was used for the PCR reaction. Plasmid pSEL1(YFP-HEX1) was constructed by inserting PCR fragment containing the *HEX1* cDNA into the *Sall* and *BamHI* sites of the pEYFP-C1 (CLONTECH) vector. Mutations in pcDNA3.1A(-)-HEX1, used for pull-down assays, were introduced with QuikChange site-directed mutagenesis according to the manufacturer's guidelines (Stratagene, La Jolla, CA), using primers listed in Supplementary data Table 2. The DNA sequences of the entire coding region of *HEX1* in the resulting constructs were verified by DNA sequencing.

Confocal laser-scanning microscopy

Murine NIH-3T3 (MMR+) and human HeLa (MMR+) cells were maintained as monolayer cultures in DMEM (Gibco, Life Technologies). HCT116 (hMLH1-) and HCT116+chr3 (MMR+) were maintained in McCoy's 5A medium (Gibco BRL, Life Technologies). All cell lines were supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/ml) and streptomycin (50 µg/ml) (Gibco BRL, Life technologies). HCT116+chr3 cells were supplemented with 400 µg/ml G418. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂. Transient transfections of YFP-hEXO1 and mutant derivatives were performed with LipofectAMINE2000 (Life Technologies Inc., USA) according to the manufacturer's instructions. Briefly, 3 × 10⁴ cells/cm² were seeded on glass plates 24 h prior to transfection incubated overnight at 37°C in a humidified 95% air–5% CO₂ atmosphere. Cells were transfected with 2 µg/ml of the relevant plasmid and left for 24–48 h before localization of YFP proteins were examined with a confocal Zeiss LSM510 microscope, as described in (31).

Western blot analysis of hEXO1 mutant proteins

For the western blot analysis, 8 × 10⁵ exponentially growing NIH-3T3 cells were seeded one day prior to treatment. On the day of treatment, cells were transfected using LipofectAMINE2000 (Life Technologies Inc., USA) according to the manufacturer's instructions with 14 µg of the relevant plasmid. Cells were incubated overnight. Whole-cell extracts were prepared as described in A. Lützen *et al.* (submitted for publication) and the protein samples (12.5 µl each) were analyzed by electrophoresis on 7% SDS polyacrylamide denaturing gel. The protein bands were transferred to Hybond-P PVDF (Amersham) membranes, and the membrane probed with rabbit anti-GFP antibody (Ref 632377, dilution 1:500, B.D. Clontech). The immunostained bands were visualized using SuperSignal (Pierce, Illinois) western blotting detection system.

In vitro transcription and translation

All IVTT reactions were carried out using the TNT-coupled reticulocyte lysate system (Promega). Briefly, lysates were incubated with 1 µg of plasmid, amino acid mix lacking cysteine, ³⁵S-Cysteine (Amersham Biosciences) and T7 RNA polymerase for 90 min at 30°C according to the manufacturer's instructions. For IVTT of unlabeled proteins ³⁵S-Cysteine was substituted with

an amino acid mix lacking methionine. IVTT proteins were resolved on 12% SDS polyacrylamide gels (Cambrex Bio Science Rockland, Inc.) and exposed to X-ray films (HyperfilmTM MP, Amersham Biosciences).

GST-fusion interaction (pull-down) assay

All plasmids expressing recombinant proteins were transformed into *Escherichia coli* BL21. For the His-tagged importin αs, the bacteria were grown at 37°C and induced with 1 mM IPTG: importin α1 for 4 h, importin α3 for 6 h, and importin α7 for 2 h. The bacteria were harvested by centrifugation and resuspended in native binding buffer (20 mM sodium phosphate; 0.5 M sodium chloride, pH 7.8), with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche). The bacteria were lysed by French Press and the lysate cleared by centrifugation 6000 rpm for 15 min and filtered using a 45-µm micron filter. About 20 mM imidazole was added to the lysate before it was loaded on an Ni-column for purification by the ProBound purification system (Invitrogen). For expression of GST-tagged importin β, the culture was induced by 1 mM IPTG at OD₆₀₀ = 0.6 for 2 h at 37°C. The bacteria were harvested by centrifugation and cells were resuspended in PBS buffer containing protease inhibitors. The GST-importin β fusion protein was purified using Separose Beads 4B (Amersham Bioscience), according to the manufacturer's guidelines, briefly; 10 ml of lysate was incubated with 1 ml beads for 2 h at 4°C. Then eluted using GEB buffer. The GST-hMLH1 and GST-hMSH2 fusion proteins were purified as described earlier (10). All the recombinant proteins were stored at –80°C with 10% glycerol (Supplementary data Figure 3).

About 10 µl (1 µg) GST-hMLH1 (100 µg/ml), GST-hMSH2 (100 µg/ml) or 2 µg importin β fusion proteins were bound to 20 µl GST beads (Glutathione SepharoseTM 4B, Amersham Biosciences) prepared in a 50% slurry with binding buffer (20 mM Tris-HCl, pH 7.5, 10% glycerol, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% Tween 20 (polyoxyethylene-sorbitan monolaurate), 0.75 mg/ml BSA containing protease inhibitors by incubation for 2 h at 4°C on a rocking platform. The protein-bound beads were washed three times with binding buffer. Samples were transferred to a 5-ml tube (Falcon), diluted with binding buffer to 50 µl GST beads/ml and incubated for 30 min at 4°C on a rocking platform. IVTT products were added to the reaction mixture and incubated for 2 h at 4°C on a rocking platform. Samples were washed three times with binding buffer. If a second protein was added, this was done after the wash and together with binding buffer; diluting GST beads to 75 µl GST beads/ml. Reactions were incubated for additional 2 h at 4°C on a rocking platform. Reaction mixtures were washed three times with binding buffer before samples were resolved on 12% SDS polyacrylamide gels (Cambrex Bio Science Rockland, Inc.) and exposed to X-ray films or visualized using PhosphorImaging (STORM 840, Amersham Pharmacia Biotech).

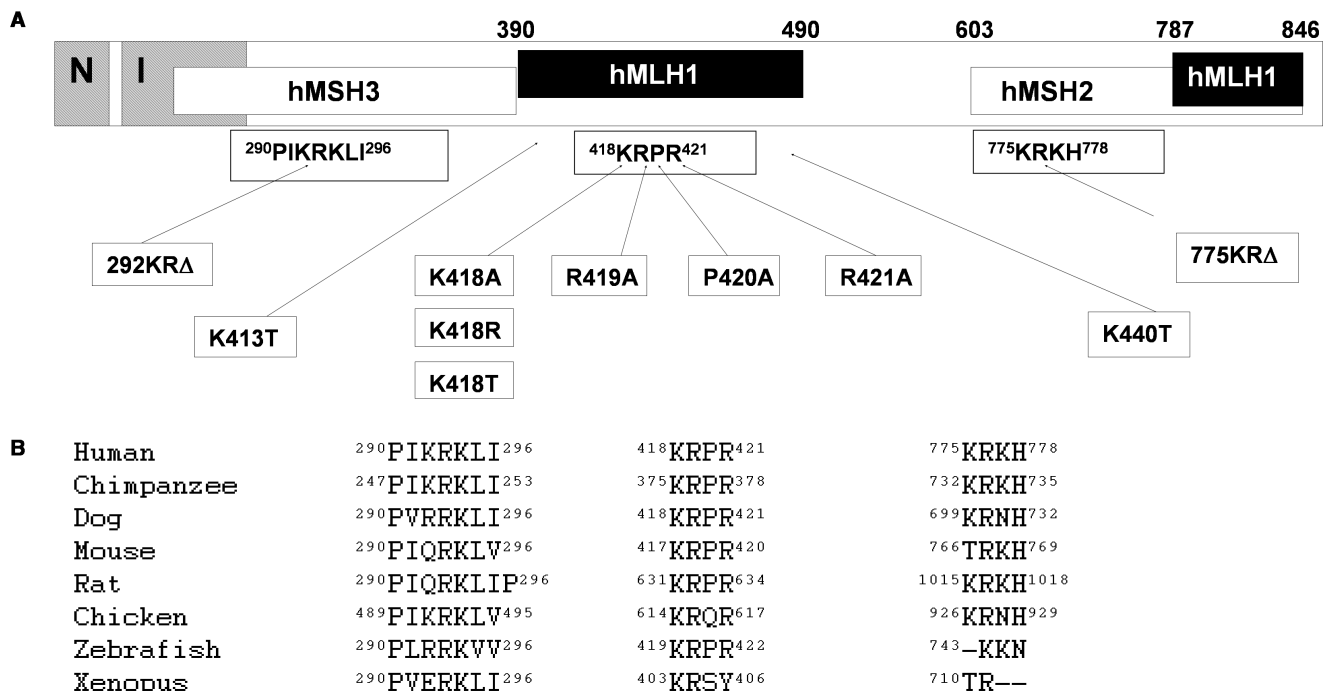


Figure 1. Exonuclease 1 gene structure. (A) The N (N-terminal) and I (internal) domains necessary for exonuclease activity as well as the regions important for protein-protein interaction with hMSH2, hMSH3 and hMLH1 are pointed out. The three putative NLS sequences ²⁹⁰PIKRKLI²⁹⁶, ⁴¹⁸KRPR⁴²¹, ⁷⁷⁵KRKH⁷⁷⁸ in hEXO1 are shown in boxes and the individual amino acids change introduced in these regions are shown below. (B) Sequence alignment of the putative NLS regions from human (Genbank: CAB45733, AAC33874, AAC63043, AAC69880, NP_003677, AAD13754, CAI15658, AAN39382, NP_006018), chimpanzee (Genbank: XP_514304), dog (Genbank: XP_547491), mouse (Genbank: NP_036142), chicken (Genbank: XP_419550), Xenopus (Genbank: AAH84102), zebrafish (Genbank: NP_998634) and rat (Genbank: XP_222932). Identical amino acids are shown in bold and numbers indicate the position of the residues in the respective proteins.

For the titration experiment shown in Figure 4 GST beads were pre-loaded with importin β and diluted to a final concentration of 75 μ l beads/ml after addition of both IVTT proteins. Importin α 3 was added and the beads incubated for 30 min at 4°C. About 10 μ l ³⁵S-labeled IVTT protein was added and incubated at 4°C. After 90 min, 5, 10, 20, 30, 50 or 100 μ l unlabeled IVTT-hEXO1 proteins were added and incubated at 4°C for 90 min. The beads were washed and analyzed as described above. The intensity of the bands was determined using IMAGEQUANT 5.2 (Molecular Dynamics).

RESULTS

Identification of hEXO1 nuclear localization signal

To identify the region(s) responsible for nuclear localization of hEXO1, we screened the amino acid sequence for possible NLS targeting signals and identified three putative NLS sequences (Figure 1). To clarify the functional significance of these three putative NLS sequences each of them were mutated in a series of YFP-hEXO1 constructs (Figure 1A) that were transiently expressed in NIH-3T3 cells. To verify that the YFP-hEXO1 mutant proteins were properly processed and not degraded, we measured the protein expression of the fluorescent fusion proteins by western blot analysis (Supplementary data Figure 1). All mutant proteins

exhibited the correct size corresponding to full-length YFP-hEXO1 fusion proteins suggesting that these YFP-fusion proteins were not degraded. The subcellular localization of the YFP fusion proteins was examined by confocal microscopy (Figure 2). As shown earlier (12) two different YFP-hEXO1 patterns were observed in cells transfected with YFP-hEXO1 — either (i) distinct nuclear foci or a (ii) diffuse nuclear staining. The diffuse nuclear staining is observed in non-S-phase cells as judged by the absence of PCNA foci (12, data not shown).

None of the examined amino acid changes in the putative NLS sequences ²⁹⁰PIKRKLI²⁹⁶ and ⁷⁷⁵KRKH⁷⁷⁸ (292KRΔG and 772KRΔG) had any effect on nuclear localization of the hEXO1 mutant proteins (Figure 2, panels 2 and 3). In contrast, when the ⁴¹⁸KRPR⁴²¹ sequence was mutated, three of the mutant proteins were localized in cytoplasm (Figure 2, panels 5–10), showing that this region is necessary for correct nuclear localization of hEXO1. It is well documented that the first lysine in the proposed consensus NLS sequence K(K/R)X(K/R) is critical for importin- α -mediated nuclear import (32–35), and substitution of the basic lysine residue to alanine, threonine or asparagine completely blocks the nuclear import of the specific protein (36). Consistent with these observations, we find that substitutions K418T and K418A in hEXO1 resulted in cytoplasmic localization of the mutant proteins. The same defect in nuclear localization was observed when we changed the basic arginine

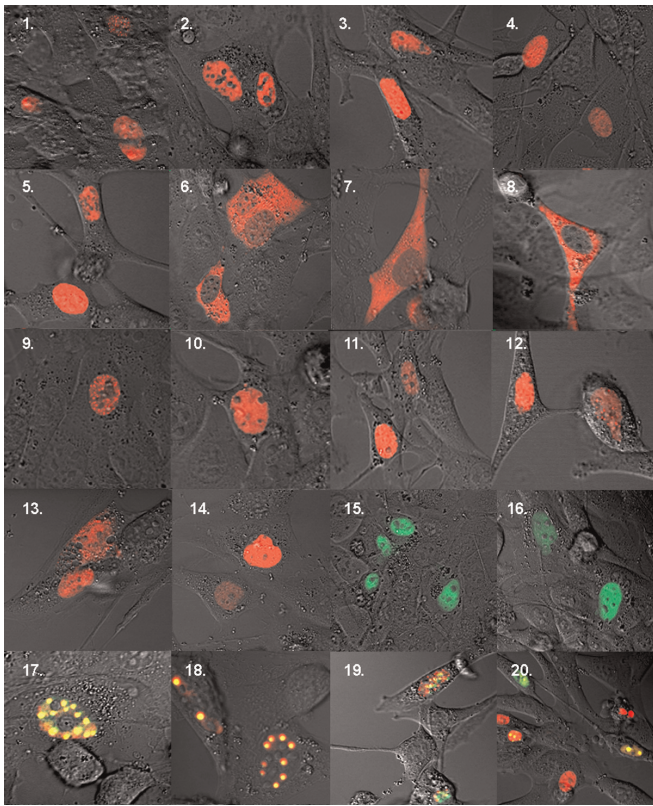


Figure 2. Nuclear localization of YFP-hEXO1 and mutant hEXO1 proteins. NIH-3T3 cells were transiently transfected with 2 µg/ml of the constructs (1) hEXO1, (2) hEXO1(292KRΔG), (3) hEXO1(772KRΔG), (4) hEXO1(K413T), (5) hEXO1(K418R), (6) hEXO1(K418A), (7) hEXO1(K418T), (8) hEXO1(R419A), (9) hEXO1(P420A), (10) hEXO1(R421A), (11) hEXO1(K440T), (12) hEXO1(L410R), (13) hEXO1(T439M), (14) hEXO1(P757L), (15) hMLH1, (16) hMSH2, (17) hEXO1 and hMLH1, (18) hEXO1 and hMSH2, (19) hEXO1(K413T) and hMLH1 and (20) hEXO1(K418A) and hMLH1. Cells were left overnight before the distribution of YFP- and CFP-tagged proteins was examined by confocal laser scanning microscopy and Nomarski contrast. YFP-tagged proteins (red), CFP-tagged proteins (green) and co-localization of fluorescent fusion proteins (yellow).

residue at position 419 of hEXO1 to alanine (Figure 2, panel 8). Along these lines, we observed normal nuclear localization and formation of nuclear foci, when we changed the proline at position 420 to alanine (P420A) as well as arginine to alanine at position 421 (R421A) (Figure 2, panels 9 and 10).

The ⁴¹⁸KRPR⁴²¹ sequence of hEXO1 shows similarity with both mono- and bipartite NLS sequences. Bipartite NLS would require a basic cluster of amino acids 10–12 amino acids upstream or downstream of the ⁴¹⁸KRPR⁴²¹ sequence. There is no indication of such a cluster upstream, but a basic cluster ⁴⁴⁰KKTKK⁴⁴⁴ is located 21 amino acids downstream of the ⁴¹⁸KRPR⁴²¹ sequence. To determine if the proposed hEXO1 NLS sequence ⁴¹⁸KRPR⁴²¹ is mono- or bipartite, we changed a single residue upstream (K413T) as well as downstream (K440T) of this sequence. None of the mutations had any effect on nuclear localization of the mutant proteins (Figure 2, panels 4 and 11) demonstrating that the

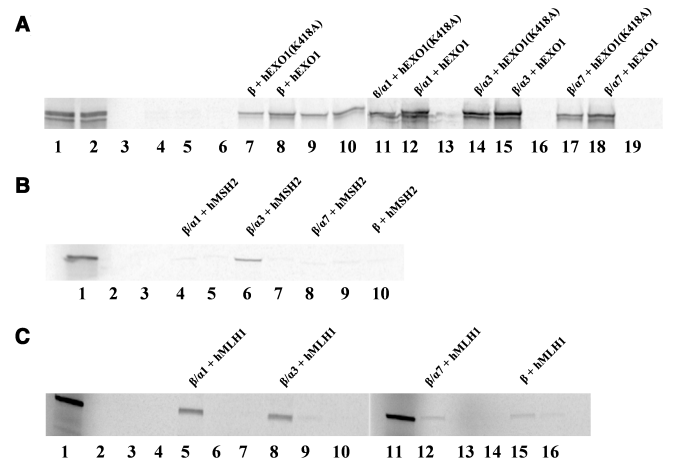


Figure 3. Interactions between importin α s and MMR proteins. (A) Lane 1: hEXO1(K418A); 2: hEXO1; 3: beads + vector; 4: beads + hEXO1(K418A); 5: beads + hEXO1; 6: *E. coli* lysate + hEXO1(K418A); 7: β + hEXO1(K418A); 8: β + hEXO1; 9: β + *E. coli* lysate + hEXO1(K418A); 10: β + *E. coli* lysate + hEXO1; 11: β + α 1 + hEXO1(K418A); 12: β + α 1 + hEXO1; 13: *E. coli* lysate + α 1 + hEXO1(K418A); 14: β + α 3 + hEXO1(K418A); 15: β + α 3 + hEXO1; 16: *E. coli* lysate + α 3 + hEXO1(K418A); 17: β + α 7 + hEXO1(K418A); 18: β + α 7 + hEXO1; 19: *E. coli* lysate + α 7 + hEXO1(K418A). (B) Lane 1: hMSH2; 2: beads + vector; 3: beads + hMSH2; 4: β + α 1 + hMSH2; 5: *E. coli* lysate + α 1 + hMSH2; 6: β + α 3 + hMSH2; 7: *E. coli* lysate + α 3 + hMSH2; 8: β + α 7 + hMSH2; 9: *E. coli* lysate + α 7 + hMSH2; 10: β + hMSH2. (C) Lane 1: hMLH1; 2: beads + vector; 3: beads + hMLH1; 4: *E. coli* lysate + hMLH1; 5: β + α 1 + hMLH1; 6: *E. coli* lysate + α 1 + hMLH1; 7: α 1 + hMLH1; 8: β + α 3 + hMLH1; 9: *E. coli* lysate + α 3 + hMLH1; 10: α 3 + hMLH1; 11: hMLH1; 12: β + α 7 + hMLH1; 13: *E. coli* lysate + α 7 + hMLH1; 14: α 7 + hMLH1; 15: β + hMLH1; 16: *E. coli* lysate + β + hMLH1.

⁴¹⁸KRPR⁴²¹ sequence is a monopartite NLS sequence of hEXO1.

Human MMR proteins recognize specific nuclear import factors

In order to characterize the basic molecular mechanism underlying import of MMR proteins into the nucleus, we examined which of the importin α s that were able to form a complex with the repair proteins. Using a pull-down assay we show that both hEXO1 and hMLH1, which contain classical NLS sequences, are able to form complexes with importin α s from all three subfamilies (α 1,3,7) as well as with importin β alone (Figure 3A and C). In contrast, we could not detect any complex formation between hMSH2 and importin α 1, α 7 or β . The MSH2 protein, which does not contain any classical NLS sequences, only formed complex with importin α 3 (Figure 3B).

We also used the pull-down assay to clarify if the cytoplasmic localization of the hEXO1 NLS mutant proteins is due to the inability of these proteins to form complexes with the importin α s. We found that the cytoplasmic hEXO1(K418A) NLS mutant protein formed complexes with all importin α s tested as well as with importin β similar to the wild-type protein (Figure 3A). This result suggests that the cytoplasmic localization of

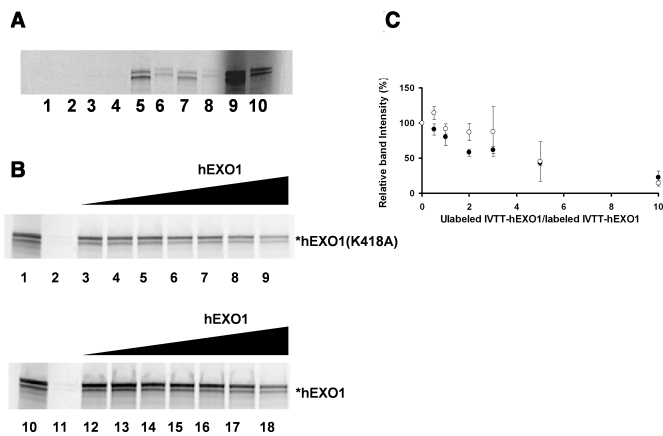


Figure 4. (A) Interaction between hEXO1(K418A) and hMLH1/hMSH2. Lane 1: beads + hEXO1(K418A); 2: *E. coli* lysate + IVTT hEXO1(K418A); 3: GST-hMSH2 + vector; 4: GST-hMLH1 + vector; 5: GST-hMSH2 + hEXO1(K418A); 6: GST-hMSH2 + hEXO1; 7: GST-hMLH1 + hEXO1(K418A); 8: GST-hMLH1 + hEXO1; 9: hEXO1(K418A); 10: hEXO1. (B) Interaction between hEXO1(K418A) and importin $\alpha 3$. Lane 1: hEXO1(K418A); 2: beads + hEXO1(K418A); 3: $\alpha 3/\beta$ + hEXO1(K418A); 4: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:0.5); 5: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:1); 6: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:2); 7: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:3); 8: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:5); 9: $\alpha 3/\beta$ + hEXO1(K418A) + unlabeled hEXO1 (1:10); 10: hEXO1; 11: beads + hEXO1; 12: $\alpha 3/\beta$ + hEXO1; 13: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:0.5); 14: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:1); 15: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:2); 16: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:3); 17: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:5); 18: $\alpha 3/\beta$ + hEXO1 + unlabeled hEXO1 (1:10). (C) Relative intensity of bands shown in (B) as a function of unlabeled IVTT-hEXO1/labelled IVTT-hEXO1/hEXO1 (K418A). Open circles: labeled hEXO1 competed with excess unlabeled hEXO1; closed circles: labeled hEXO1(K418A) competed with excess unlabeled hEXO1. Standard deviations are shown. Each data point in this figure represents the average from three independent experiments.

hEXO1 NLS mutant proteins is not caused by a deficiency of these proteins to recognize the nuclear import factors.

hEXO1 enhance nuclear foci formations of hMLH1 and hMSH2

It has been shown that complex formation between hMLH1 and hPMS2/hPMS1 is essential for correct nuclear translocation of the PMS proteins (24,25). *In vitro* results suggest that hEXO1 forms ternary complexes with both hMLH1-hPMS2 and hMSH2-hMSH6 via direct protein-protein interaction between hEXO1-hMLH1 and hEXO1-hMSH2 (12,13). These complex formations are critical for MMR activity, but it is not clear whether they play roles in subcellular localization of the MMR complexes. To investigate if hMSH2 and hMLH1 influence subcellular localization of hEXO1, we examined cells co-transfected with CFP-hMSH2 and YFP-hEXO1 as well as CFP-hMLH1 and YFP-hEXO1 (Figure 2, panels 17 and 18). As shown earlier two different patterns were observed in cells transfected with YFP-hEXO1 (i) distinct nuclear foci or a (ii) diffuse nuclear staining (13, data not shown; Figure 2). In contrast, we did not observe any discrete nuclear punctates in cells transfected solely with

Table 1. hEXO1 nuclear localization and formation of nuclear speckles in the presence of hMLH1 or hMSH2

Vector ^a	Nuclear localization ^b	Nuclear Speckles ^b
pYFP-hEXO1	92 (5)	15 (5)
pCFP-hMLH1	92 (7)	4 (6)
pCFP-hMSH2	95 (7)	2 (5)
pYFP-hEXO1(K413T)	94 (8)	9 (8)
pYFP-hEXO1(K418A)	0 (0)	0 (0)
pYFP-hEXO1 + pCFPhMLH1	98 (5)	45 (12)*
pYFP-hEXO1 + pCFPhMSH2	100 (0)	67 (10)*
pYFP-hEXO1(K413T) + pCFP-hMLH1	100 (0)	39 (5)*
pYFP-hEXO1(K413T) + pCFP-hMSH2	100 (0)	55 (16)*
pYFP-hEXO1(K418A) + pCFP-hMLH1	50 (18)* [#]	31 (13)
pYFP-hEXO1(K418A) + pCFP-hMSH2	76 (17)* [#]	60 (10)*

^aThe indicated CFP- or YFP-tagged constructs were transfected into NIH-3T3 cells and examined by confocal microscopy. ^bAt least five consecutive visual fields corresponding to approximately 150 cells were counted. The results are stated as mean and SD (in brackets). * $P < 0.05$ (Student t-test, two tailed, equal variance). [#]In all cells, cytoplasmic YFP-hEXO1(K418A) and CFP-hMLH1 or CFP-hMSH2 were observed and the corresponding number indicates the percentage of cells with increased levels of speckled or diffuse nuclear staining compared to controls (YFP-hEXO1(K418A)).

CFP-hMLH1 or CFP-hMSH2 (Figure 2, panels 15 and 16). When cells were co-transfected with either YFP-hEXO1 and CFP-hMSH2 or YFP-hEXO1 and CFP-hMLH1, the formation of nuclear foci was increased (Figure 2, Table 1); indicating that complex formation between hEXO1 and hMLH1 or hMSH2 affects the distribution of the MMR protein in nucleus. The hEXO1 protein is not critical for nuclear import of hMLH1 and hMSH2. However, since NIH-3T3 cells express MMR proteins and it has been shown that murine homologs can form complexes with the human proteins (24) it is possible that the endogenously expressed MMR proteins in NIH-3T3 cells could affect the distribution of hEXO1. Therefore, we expressed YFP-hEXO1 in the human cell lines HeLa (MMR proficient), HCT116 (deficient in hMLH1) as well as in HCT116 + chromosome 3 (proficient in hMLH1). We observe the same localization pattern as in NIH-3T3 cells for all cell lines tested (data not shown).

To address whether the presence of hMLH1 and hMSH2 in the nucleus promotes the localization of hEXO1 in foci, we repeated the experiment shown in Figure 2 (panels 17 and 18) using the NLS-deficient hEXO1(K418A) as well as the NLS-proficient hEXO1(K413T) proteins (Table 1). The hEXO1(K418A) mutant protein is unable to localize to nucleus as shown in Figure 2 (panel 6) and, therefore, we did not expect enhanced nuclear foci formation in cells co-expressing YFP-hEXO1(K418A) with either CFP-hMLH1 (Figure 2, panel 20; Table 1) or CFP-hMSH2 (data not shown; Table 1). However, in contrast to cells transfected with YFP-hEXO1(K418A) alone, a significant portion of this fusion protein was however visible in the nucleus and co-localized with CFP-hMLH1 in discrete nuclear punctates (Figure 2, panel 20; Table 1). The same subcellular localization pattern was observed when

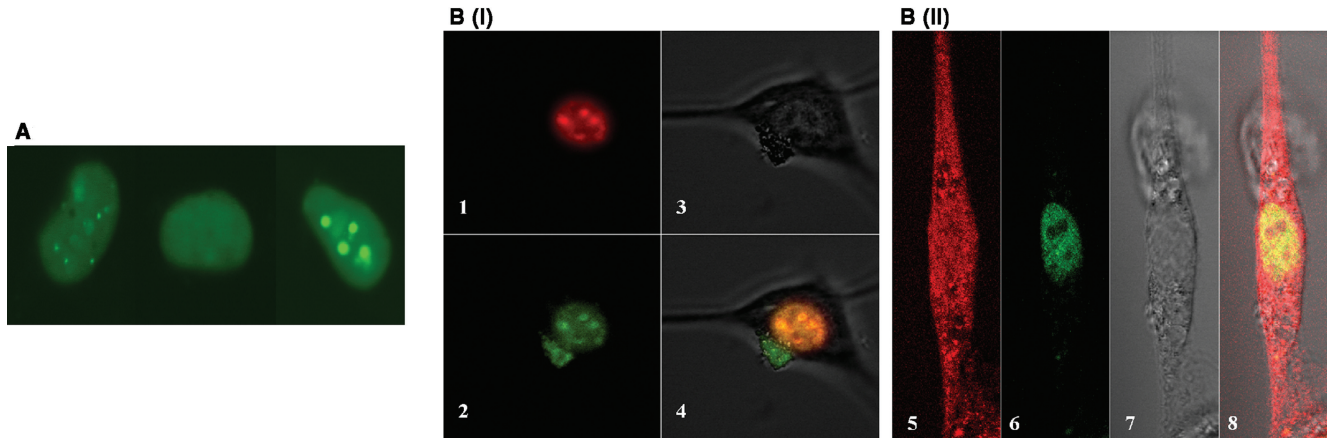


Figure 5. Subcellular localization of HEX1. Fusion proteins were visualized by confocal imaging. (A) NIH3T3 cells transfected with YFP-HEX1. (B) NIH-3T3 cells co-transfected with YFP-HEX1 and CFP-PCNA. (I) Transfected cell in S-phase, 1: CFP-PCNA; 2: YFP-HEX1; 3: Normaski image; and 4: Overlay of CFP-PCNA and YFP-HEX1. (II) Transfected cell not in S-phase, 5: CFP-PCNA; 6: YFP-HEX1; 7: Normaski image; and 8: Overlay of CFP-PCNA and YFP-HEX1.

YFP-hEXO1(K418A) was co-transfected with CFP-hMSH2 although the nuclear foci formation was more pronounced for the CFP-hMLH1 co-transfections (data not shown). Interestingly, we found that the NLS-defective YFP-hEXO1(K418A) mutant proteins only translocated to the nucleus when hMSH2 or hMLH1 formed nuclear foci. In cells showing diffuse nuclear staining of hMSH2 or hMLH1, the hEXO1(K418A) mutant protein remained in cytoplasm (data not shown). Furthermore, we did not observe any hEXO1-N (hEXO1 NH₂-terminal YFP-fusion protein that does not contain the NLS as well as the hMLH1-interacting domain and which localizes to cytoplasm (13)) in nucleus when this fusion protein was co-expressed with CFP-hMLH1 (data not shown). It has been shown earlier that hEXO1 does not interact with hPMS2 (10). We also found that in cells co-expressing YFP-hEXO1(K418A) and CFP-hPMS2 the NLS-defective hEXO1 mutant protein localized in cytoplasm (data not shown). Our results suggest that the NLS-defective hEXO1 mutant proteins are able to form complexes with hMSH2 and hMLH1 but not hPMS2.

The hEXO1 NLS sequence is located in the domain required for interaction with hMLH1 (Figure 1), thus we further investigated the ability of the hEXO1(K418A) protein to form complex with hMLH1 and hMSH2 in the *in vitro* pull-down assay. We show that hEXO1(K418A) can form a complex with both hMLH1 as well as hMSH2 in this assay (Figure 4A). These results suggest that either (i) hMSH2 and hMLH1 are able to co-translocate hEXO1(K418A) into nucleus in the lack of a functional hEXO1 NLS sequence or that (ii) YFP-hEXO1(K418A) fusion protein is quickly exported to cytoplasm if it is not in complex with hMLH1 or hMSH2. A third (iii) possibility would be that the complex formation between hEXO1(K418A) and importin α is less efficient than the similar protein-protein interaction between the importin α and the wild-type hEXO1 proteins resulting in cytoplasmic retention of the hEXO1(K418A) mutant protein. In order to test the third possibility,

we performed a pull-down assay where we labeled either hEXO1(K418A) or hEXO1 and incubated these proteins with importin α 3/ β complex. To compare complex formations between importin α 3/ β and hEXO1(K418A) or hEXO1, we added increasing amounts of unlabeled IVTT-hEXO1 or purified hEXO1 to compete for binding with the labeled proteins (Figure 4B, Supplementary data Figure 4B) or included the competitor during (rather than after) complex formation (Supplementary data Figure 4A). We did not find any major difference in complex formation between importin α 3/ β and the hEXO1 proteins suggesting that the hEXO1 NLS mutant proteins do not have lower affinity for the nuclear import factors (Figure 4C).

Subcellular localization of HEX1(hEXO1a)

The *hEXO1* gene generates two splice variants, *HEX1* (*hEXO1a*) and *hEXO1b*, which encode polypeptides of 803 and 846 amino acids, respectively (2). Both isoforms contain the exonucleolytic domain and so far no biological or biochemical differences between the two proteins have been reported. We have shown earlier that the COOH-terminal region of hEXO1 is essential for correct nuclear localization as well as for the co-localization of hEXO1b with PCNA during S-phase (10,13). Therefore, it is possible that the two different hEXO1 splice variants differ in subcellular localization pattern. The short splice variant of the human exonuclease 1, HEX1 (hEXO1a), differs from hEXO1b by lacking the last 43 COOH-terminal amino acids. Since subcellular localization of HEX1 and co-localization with PCNA have not been addressed earlier, we examined if there were any differences in the subcellular localization of HEX1 and hEXO1b. Results presented in Figure 5 demonstrate that like hEXO1b the subcellular localization of HEX1 is restricted to the nucleus and that HEX1 co-localizes with PCNA. Moreover, we infer that both splice variants of hEXO1 may be involved in processes where interactions with PCNA are required, i.e. DNA replication and/or MMR. Although the COOH-terminal part of hEXO1b is

crucial for subcellular localization, the 43 COOH-terminal amino acids have no influence on the subcellular localization of hEXO1.

Subcellular localization and protein complex formation of hEXO1 variant proteins

Deficiency in MMR can be a result of both defective protein–protein interaction (complex formation) and defective subcellular localization of MMR proteins. In order to characterize variants of the *hEXO1* gene, we included two variants, V27A and L410R, which have been identified only in HNPCC patients, and could therefore represent pathogenic mutations. The remaining R354H, T439M, K589G, G670E and P757L variants, were identified with same frequency in patients and control individuals (15) and may be considered as naturally occurring polymorphisms, although the hEXO1 genotypes T439M and P757L have been shown to be correlated with development of colorectal cancer (37). None of the amino acids substituted in the hEXO1 variants are located in the putative NLS sequences. However, L410R is located in the vicinity of the functional NLS sequence ⁴¹⁸KRPR⁴²¹ and T439M near the basic ⁴⁴⁰KKTKK⁴⁴⁴ cluster located 21 amino acids downstream of the ⁴¹⁸KRPR⁴²¹ sequence. Figure 2 shows that the localization of YFP-hEXO1 variant proteins is restricted to the nucleus and resembles the pattern observed for wild-type YFP-hEXO1.

We have shown earlier that hEXO1 can form ternary complexes with both hMutS α and hMutL α and that these protein–protein interactions control both the biochemical activity of the proteins as well as the subcellular localization of the exonuclease (12,13; Figure 2; Table 1). In order to investigate if the hEXO1 variants act differently regarding complex formation with other MMR proteins, we investigated the ability of the variants to interact with hMSH2, hMLH1 as well as hMutS α and hMutL α (Figure 6). Our results show that all HEX1 variant proteins interact with GST-hMLH1 as well as with GST-hMSH2 (Figure 6A and B). Furthermore, the variants HEX1-L410R and HEX1-T439M interact with hMutS α and hMutL α suggesting that these proteins can form ternary complexes with hMutS α and hMutL α like the wild-type protein (Figure 6C and data not shown). Under the experimental conditions employed, the GST-MMR proteins, which are bound to the beads, are saturated with the first added protein (hMSH6 or hPMS2) before the second protein (hEXO1) is added to the reaction mixture. However, it cannot be completely excluded that some of the pre-incubated protein may dissociate and become exchanged with the second protein. In conclusion, our results indicate that if the described HEX1 variants are pathogenic this can neither be explained by defective hMLH1 or hMSH2 protein interaction nor abnormal subcellular localization.

DISCUSSION

In this article, we examined the nuclear translocation of hEXO1. These types of studies are important for

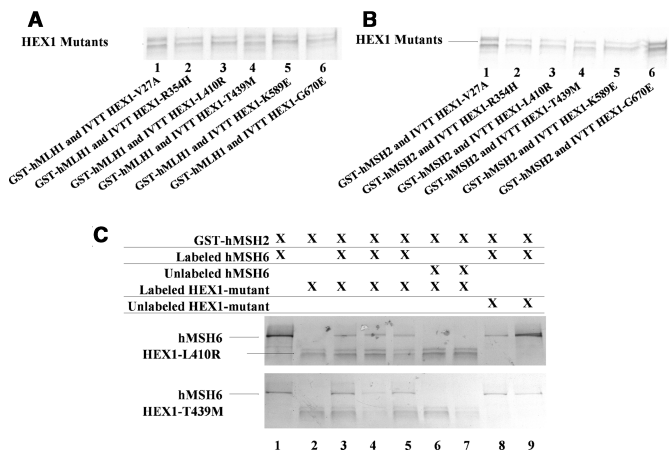


Figure 6. Interaction between hEXO1 variants and hMLH1, hMSH2 and hMSH2/hMSH6. (A) Lane 1: hMLH1 + HEX1-V27A; 2: hMLH1 + HEX1-R354H; 3: hMLH1 + HEX1-L410R; 4: hMLH1 + HEX1-T439M; 5: hMLH1 + HEX1-K589E; 6: hMLH1 + HEX1-G670E. (B) Lane 1: hMSH2 + HEX1-V27A; 2: hMSH2 + HEX1-R354H; 3: hMSH2 + HEX1-L410R; 4: hMSH2 + HEX1-T439M; 5: hMSH2 + HEX1-K589E; 6: hMSH2 + HEX1-G670E. (C) Lane 1: hMSH2 + hMSH6; 2: hMSH2 + HEX1; 3: hMSH2 + hMSH6 + HEX1 (hMSH6 and HEX1 were added to hMSH2 simultaneously); 4: hMSH2 incubated for 2h with HEX1 followed by 2h incubation with hMSH6; 5: hMSH2 incubated for 2h with hMSH6 followed by 2h incubation with HEX1; 6: hMSH2 incubated for 2h with HEX1 followed by 2h incubation with unlabeled hMSH6; 7: hMSH2 incubated for 2h with unlabeled hMSH6 followed by 2h incubation with HEX1; 8: hMSH2 incubated for 2h with unlabeled HEX1 followed by 2h incubation with hMSH6; 9: hMSH2 incubated for 2h with hMSH6 followed by 2h incubation with unlabeled HEX1. Control reactions were performed as described in (12) (data not shown). Samples were resolved on 12% SDS-PAGE gel and imaged by exposure to X-ray films.

(i) understanding the basic molecular mechanisms underlying MMR as well as for (ii) identification of the multiple ways by which the MMR pathway can be inactivated. The latter may have implications for diagnosis of HNPCC.

As a first step to understand the molecular mechanisms underlying subcellular movements of MMR proteins, we investigated the function of three putative NLS sequences ²⁹⁰PIKRKLI²⁹⁶, ⁴¹⁸KRPR⁴²¹ and ⁷⁷⁵KRKH⁷⁷⁸ in hEXO1. None of these are located in the N and I regions required for exonuclease activity, but they are conserved among hEXO1 proteins of higher eukaryotes (Figure 1B) suggesting a biological role of these sequences. Mutations in critical residues of the ⁴¹⁸KRPR⁴²¹ sequence in hEXO1 resulted in loss of nuclear translocation—in particular residues K418 and R419 are essential for nuclear targeting. These lysine and arginine residues are included in a consensus NLS sequence that presumably binds to importin α 1, 3 and 7. At least two types of NLS sequences have been identified namely the mono- and bipartite NLS. The basic ⁴⁴⁰KKTKK⁴⁴⁴ cluster located 21 amino acids downstream of the ⁴¹⁸KRPR⁴²¹ sequence, could be part of a major binding site. This would imply that the K418 residue takes part in a minor binding site of the bipartite NLS sequence. However, mutations in the minor binding site

of a bipartite sequence generally do not have great impact on protein translocation (38). Furthermore, the distance between the putative major and minor binding site in hEXO1 is larger than the expected 10–12 amino acids and the K440T mutant protein localizes similar to wild-type hEXO1. Taken together our results show that the ⁴¹⁸KRPR⁴²¹ sequence is a monopartite NLS sequence.

The hMSH2 protein does not contain classical NLS sequences (data not shown) and, therefore, it is possible that this MMR protein translocates into the nucleus in complex with other proteins similar to hMutL α and hMutL β (24–26). The fact that hMSH2 does not contain a classical NLS is surprising since this MMR protein localizes to the nucleus when overexpressed as a CFP-hMSH2 fusion protein (Figure 2) and it forms a complex with nuclear import factors importin α 3/ β *in vitro* (Figure 3). Interestingly, our results show that unlike hEXO1 and hMLH1, which both recognize several importin α s, hMSH2 specifically forms a complex with importin α 3. In order to identify the sequence responsible for nuclear translocation of hMSH2, we identified two mutations P622L and C697F in hMSH2 that result in cytoplasmic localization of the proteins (A. Lützen *et al.*, submitted for publication). However, when these mutant proteins were tested in the pull-down assay for interaction with importin α 3/ β , they formed complexes with the nuclear import factors similar to the wild-type protein (Supplementary data Figure 2). These results suggest that the mechanisms underlying nuclear import of the individual MMR proteins are different, which could play an important biological role in regulation of MMR activity.

Protein–protein interactions between hEXO1, PCNA, RPA, RFC and hMutS α and hMutL α are important for regulation of overall repair activity (4). However, it is unclear if these protein–protein interactions are also important for subcellular localization of the MMR proteins. We therefore addressed whether the co-expression of hEXO1 and hMLH1 or hMSH2 would alter the subcellular distribution of these proteins. A clear increase in the formation of nuclear foci was observed upon co-transfection indicating that complex formation facilitates proper localization (Figure 2; Table 1). This was even observed with the hEXO1(K418A) mutant protein, which alone is unable to localize to nucleus. It was further demonstrated that hEXO1(K418A) forms complex with hMLH1 and hMSH2. We measured co-localization of PCNA with hEXO1 as well as PCNA with hMLH1 (data not shown) and we did not detect any increase in the percentage of S-phase cells suggesting that co-expression of hEXO1 and hMLH1 does not stimulate cell proliferation. We have shown earlier (13) that not all foci represent replication foci and, therefore, we cannot conclude that the punctuate staining observed in this work represents replication factories. Examples of a similar piggy-back mechanism where NLS-dysfunctional proteins can be translocated to the nucleus through complex formation with proteins containing functional NLS sequences are known from other repair proteins such as for the KU70/80 kinase and MRN (hMRE11-hRAD50-NBS1) complexes, which are involved in double-stranded

break repair (39,40). Taken together the results support the idea that hEXO1, hMLH1 and hMSH2 form complexes, which under certain conditions are imported together and that redundant NLS import signals in the proteins may safeguard import and MMR activity.

Mutations located outside the NLS sequence could alter the protein conformation in a way that makes the NLS sequence inaccessible to importin binding. Therefore, we found it relevant to examine sequence variations in the *hEXO1* gene from cancer patients in or near the ⁴¹⁸KRPR⁴²¹ sequence and determine the subcellular localization of these mutant proteins. Our results show that all hEXO1 variant proteins examined localized to the nucleus similar to the wild-type protein. The L410R variation in particular has been found in atypical HNPCC patients (15). The mutant is translocated to the nucleus similar to wild-type hEXO1 (Figure 2), but it is defective in exonuclease activity despite the fact that the mutation is located within the hEXO1-hMLH1-binding domain and does not seem to have any effect on complex formation between these two proteins (41, Figure 6). Overall, these results suggest that the hEXO1-L410R mutant protein could act as a potential dominant negative resulting in defective MMR activity in individuals carrying this allele.

Finally, our results demonstrate no functional difference between HEX1 and hEXO1b with respect to subcellular localization and co-localization with PCNA. It can therefore be concluded that the 43 COOH-terminal amino acids are not involved in nuclear localization signaling. The HEX1 protein is capable of interacting with both hMLH1 and hMSH2 (Figure 6) and it has been established that HEX1 is capable of forming a ternary complex with hMutL α and hMutS α (data not shown; Figure 6), as demonstrated to apply for hEXO1b (11,12). The results presented demonstrate that all six HEX1 mutants V27A, R354H, L410R, T439M, K589E and G670E are able to interact with both hMLH1 and hMSH2 and in addition, that amino acid substitutions L410R and T439M do not interfere with the ability of HEX1 to participate in ternary complex formation with hMutL α or hMutS α .

In conclusion, we have identified the sequence ⁴¹⁸KRPR⁴²¹, which exhibit strong homology to other monopartite NLS sequences to be responsible for correct nuclear localization of hEXO1. We have shown that hEXO1 and hMLH1 are recognized by nuclear import factors importin α 1,3,7/ β whereas hMSH2 is specifically recognized by importin α 3/ β . These results suggest differences in the mechanisms underlying nuclear translocation of MMR proteins. We also showed that co-expression of hEXO1 NLS mutant proteins with either hMLH1 or hMSH2 restored nuclear localization of hEXO1 NLS mutant proteins. Our results suggest that hEXO1, hMLH1 and hMSH2 form complexes, which are imported to the nucleus together. However, complex formations between these MMR proteins are not essential for nuclear translocation implying that redundant NLS import signals in the proteins may ensure nuclear import and thereby MMR activity. It can be speculated that repair proteins that either lack a functional NLS

or do not contain sequences that bind importin $\alpha\beta$ form complexes in the cytoplasm before nuclear import. Such mechanism would safeguard nuclear import of proteins since karyophilic proteins would increase nuclear localization upon complex formation, instead of individually competing for interaction with the NPC. Therefore, it would be interesting to examine if complex formation between MMR proteins in cytoplasm plays a similar role in subcellular localization. However, it is not clear whether the piggy-back translocation we observed acts as a backup mechanism or if it is essential for the regulation of MMR activity *in vivo*. But the importance of complex formation between MMR proteins has been illustrated by the finding that some single amino acid substitutions in both hMLH1 and hMSH2 found in HNPCC patients disrupt complex formations and show defective nuclear localization (42; A. Lützen *et al.*, submitted for publication). None of the hMLH1 mutations were located near its functional NLS sequence. Several MMR proteins interact with proteins involved in other repair pathways. Therefore, it could be speculated that proteins involved in other repair systems could be involved in the translocation of MMR proteins, thus, linking nuclear translocation of repair systems.

SUPPLEMENTARY DATA

Supplementary data is available at NAR online.

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