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Degadration of mismatch repair hMutSα heterodimer by the ubiquitin-proteasome pathway

Hélène Hernandez-Pigeon[a], Guy Laurent[a,b], Odile Humbert[c], Bernard Salles[c], Dominique Lautier[a,*]

[a] *INSERM U563, Centre de Physiopathologie Toulouse Purpan, Institut Claudius Regaud, 20 rue du Pont Saint-Pierre, 31052 Toulouse, France*
[b] *Service d'Hématologie, Hôpital Purpan, Place du Dr. Baylac, 31059 Toulouse, France*
[c] *Institut de Pharmacologie et Biologie Structurale, UMR 5089 CNRS, 205 route de Narbonne, 31077 Toulouse, France*

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Abstract Mismatch repair plays a critical role in genome stability. This process requires several proteins including hMSH2/hMSH6 (hMutSα) heterodimer involved in the first stage of the process, the mispair recognition. We previously reported that in U937 and HL-60 cell lines, hMSH2 and hMSH6 protein expression was much lower than that in HeLa and KG1a cells. Here, we showed that the decreased expression of hMutSα results from differences in the degradation rate of both proteins by the ubiquitin-proteasome pathway. Our data suggest that in human cell lines, ubiquitin-proteasome could play an important role in the regulation of hMutSα protein expression, thereby regulating mismatch repair activity.

Key words: MSH2; MSH6; Ubiquitination; Protein stability; Mismatch repair; Proteasome

1. Introduction

Cancer cells are characterized by multiple genetic abnormalities that could interfere with cell growth, differentiation, motility and sensitivity to chemotherapy. These observations suggest that the mechanisms controlling genetic stability could be altered in cancer cells [1]. An example of tumors displaying mismatch repair (MMR) dysfunction is given by inherited familial cancer syndrome of hereditary non-polyposis colon cancers [2]. MMR plays an important role in the maintenance of genomic integrity by correcting replicative mismatches (nucleotide mispairs, insertion/deletion loops) that escape DNA polymerase proofreading [3]. MMR activity begins with mismatch recognition either by hMutSα, a heterodimer of hMSH2 and hMSH6 proteins, or by hMutSβ (a heterodimer of hMSH2 and hMSH3). A second complex hMutLα (a heterodimer of hMLH1 and hPMS2) that facilitates mismatch correction is then recruited. Strand discrimination and degradation of the strand carrying the wrong base occur prior to DNA strand gap repair synthesis. Defective MMR is characterized by the production of multiple replication errors in repetitive DNA sequences (microsatellites), leading to microsatellite instability (MSI), and an elevated rate of spontaneous mutations, resulting in a mutator phenotype.

MSI is related to absent or weak expression of MMR proteins that does not always result from MMR gene mutation or promoter methylation [4–6]. Previous studies have established that hMutSα proteins can be regulated at the transcriptional level and that AP1 and p53 are involved in gene regulation [7–9]. However, hMSH2 and hMSH6 protein expression does not always correlate with mRNA levels [10,11]. One possible mechanism for regulation of protein expression is proteolysis by the ubiquitin-proteasome (Ub-proteasome) pathway [12]. This process results from the covalent conjugation of Ub to proteins, catalyzed by a family of Ub-conjugating and Ub-ligating enzymes. The ubiquitinated proteins can be further degraded by the 26S proteasome constituted by a catalytic complex (20S) and two regulator complexes (19S). Ub-proteasome regulates several DNA repair processes by degrading various proteins, including p53 [13], XPC [14], and MGMT [15].

We showed recently that different hMutSα protein levels were found in various cell lines [16]. In this paper, we investigated whether the Ub-proteasome pathway could be involved in the regulation of hMutSα protein expression.

2. Materials and methods

2.1. Cells and chemicals

U937 (monocytic), HL-60 (myelocytic), KG1a (promyeloblastic), HeLa (epithelial), and MRC-5 (fibroblast) cell lines were obtained from the ATCC (Rockville, MD, USA). U937 and HL-60 cell lines were grown in RPMI 1640 containing 10% fetal calf serum (FCS). KG1a cells were grown in IMDM containing 20% FCS. HeLa and MRC-5 cell lines were grown in MEM containing 10% FCS. Culture media were supplemented with 2 mM glutamine, streptomycin (100 μg/ml) and penicillin (200 U/ml). All other reagents were purchased from Sigma-Aldrich (St Quentin-Fallavier, France).

2.2. Immunoprecipitation and Western blot analysis

For immunoprecipitation, cell extracts were incubated overnight at 4°C with anti-MSH6 (Transduction Laboratories, Lexington, KY, USA), anti-MSH2 (Oncogene Research Products, Boston, MA, USA) or anti-Ub (Zymed, San Francisco, CA, USA) antibodies. Samples were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes and probed with anti-MSH2 (1 μg/ml), anti-MSH6 (1 μg/ml), anti-Ub (1 μg/ml) or anti-β-actin (2 μg/ml) (Chemicon, Temecula, CA, USA) antibodies. Detection was performed with the enhanced chemiluminescence system ECL Kit (Amersham Pharmacia

*Corresponding author. Fax: (33)-561-42 46 06.
E-mail address: lautier@icr.fnclcc.fr (D. Lautier).

Abbreviations: CHX, cycloheximide; DCIC, dichloroisocoumarin; MCA, 7-amino-4-methylcoumarin; MMR, mismatch repair; MSI, microsatellite instability; RRL, rabbit reticulocyte lysate; TPCK, 1-chloro-3-tosylamido-4-phenyl-2-butanone; Ub, ubiquitin

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Table 1
Oligonucleotide primer sequences for real-time quantitative PCR

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)	T_m	E
hMSH2	AAGGCTTCTCCTGGCAATCT	CACAACACCAATGGAAGCTG	81	80.9	94.4
hMSH6	AGACTTGGTGCCTCAGACAGA	TCATCCACAAGCACCAGAGA	116	81.8	93.9
β-Actin	TCCCTGGAGAAAGAGCTACGA	AGGAAGGAAGCCTGGAAGAG	98	87.2	92.3

The length of the specific amplification products, their approximate melting temperature (T_m) (°C) and their PCR efficiency (E) are indicated.

Biotech, Saclay, France). The quantification of protein expression levels was done by the NIH Image software.

2.3. Real-time quantitative polymerase chain reaction (PCR)

Total cellular RNA was extracted with TRIzol®. Reverse transcription of total RNA was performed with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ Life Technologies, Cergy Pontoise, France). Forward and reverse primers were designed using Primer Input 3 for each gene of interest (Table 1). Real-time PCR was performed using an iCycler thermal cycler (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions. Reactions were performed with 0.3 μM primers. Nucleotides, Taq DNA polymerase, and buffer were included in SYBR Green JumpStart™ Taq ReadyMix™ for quantitative PCR. cDNA amplification consisted of one cycle at 95°C for 1 min 30 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. The threshold cycle (C_T) values were determined by iCycler software (Bio-Rad) and the quantification data were analyzed following the $\Delta\Delta C_T$ method using β-actin as reference. We have checked that PCR efficiency (E) of the amplification was similar whatever the primers and we calculated the relative amount (RA): $RA = (1+E)^{-\Delta\Delta C_T}$.

2.4. In vitro determination of proteasome activity

The in vitro activity of the 20S proteasome was determined according to Grune et al. [17]. Cells were re-suspended in phosphate-buffered saline containing 0.1% Triton X-100 and 0.5 mM dithiothreitol (DTT), homogenized and used immediately for measuring the enzymatic activity. The assay mixture contained 50 mM Tris–HCl pH 7.8, 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM DTT, 250 μM sLLVY-MCA and 50 μg of proteins. After 1 h at 37°C, the reaction was stopped by adding 1 ml of 0.2 M glycine buffer pH 10. The fluorescence of the proteolytically released 7-amino-4-methylcoumarin (MCA) was excited at 370 nm and the emission was recorded at 460 nm.

2.5. Degradation assay

Degradation of hMutSα proteins was determined on cell homogenates using minor modifications of the method described previously [18]. Briefly, cell extracts were prepared by addition of lysis buffer. Anti-MSH2 and anti-MSH6 immunocomplexes recovered by binding to protein G-Sepharose beads were used as substrates for in vitro degradation. The degradation reaction included immunoprecipitates, 100 μg of rabbit reticulocyte lysate (RRL) as a source of Ub-proteasome factors, and 2 mM ATP, 1 mM creatine phosphate, 25 U/ml creatine phosphokinase and 0.1 μg/ml Ub. Where indicated MG132 (50 μM) was added. The degradation mixture was incubated for 90 min at 30°C. The samples were eluted by boiling in 1×Laemmli buffer, and separated by a 7.5% SDS–PAGE, transferred onto nitrocellulose membranes, and incubated with anti-MSH2 and anti-MSH6 antibodies.

3. Results

3.1. hMSH2 and hMSH6 expression in human cells

We compared hMSH2 and hMSH6 protein levels in different cell lines (U937, HL-60, KG1a, MRC-5 and HeLa cells). hMSH2 and hMSH6 protein expression was very low in U937 and HL-60 cells, compared to that in KG1a, MRC-5 and HeLa cells (Fig. 1A). We first investigated the possibility of a transcriptional regulation, but real-time quantitative PCR analyses revealed no significant differences in hMSH2 and hMSH6 mRNA levels between the different cell lines (Fig. 1B).

Thus, the differences in hMutSα protein expression result from post-transcriptional processes and we hypothesized an involvement of differences in protein stability in the different cell lines.

3.2. hMSH2 and hMSH6 protein stability in human cell lines

We determined hMSH2 and hMSH6 protein stability in U937, HL-60, KG1a, MRC-5 and HeLa cell lines, following inhibition of de novo protein synthesis resulting from cell treatment with cycloheximide (CHX) (Fig. 2). In U937 and HL-60 cell lines, hMSH2 and hMSH6 protein levels markedly decreased with time. In contrast, only a weak degradation was observed in KG1a and HeLa cell lines while the degradation rate in MRC-5 cells was in between (Fig. 2A). Densitometric analysis showed that half-lives of hMutSα components in U937 cells were about 14 h and 12.5 h, respectively and even shorter in HL-60 cells (about 8 h and 7.5 h, respectively) compared to KG1a, MRC-5 and HeLa cells (longer than 20 h) (Fig. 2B). In all cell lines, the half-lives of the two proteins were strongly correlated.

These results showed that the hMutSα degradation rates were cell-dependent and inversely correlated with hMutSα protein expression levels.

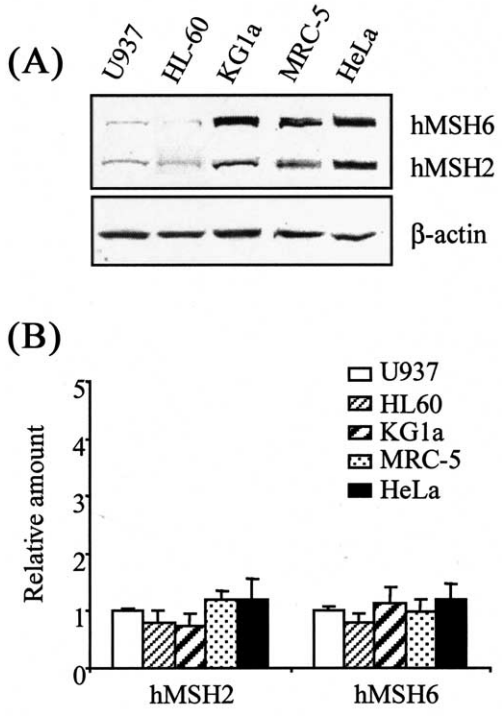

Fig. 1. A: Expression of hMSH2 and hMSH6 proteins in different human cell lines. β-Actin was used as loading control. B: Expression of hMSH2 and hMSH6 mRNA measured by real-time quantitative PCR in human cell lines. The data are expressed as the relative amounts compared with values from U937 cells. The results are the mean (±S.D.) of three independent experiments.

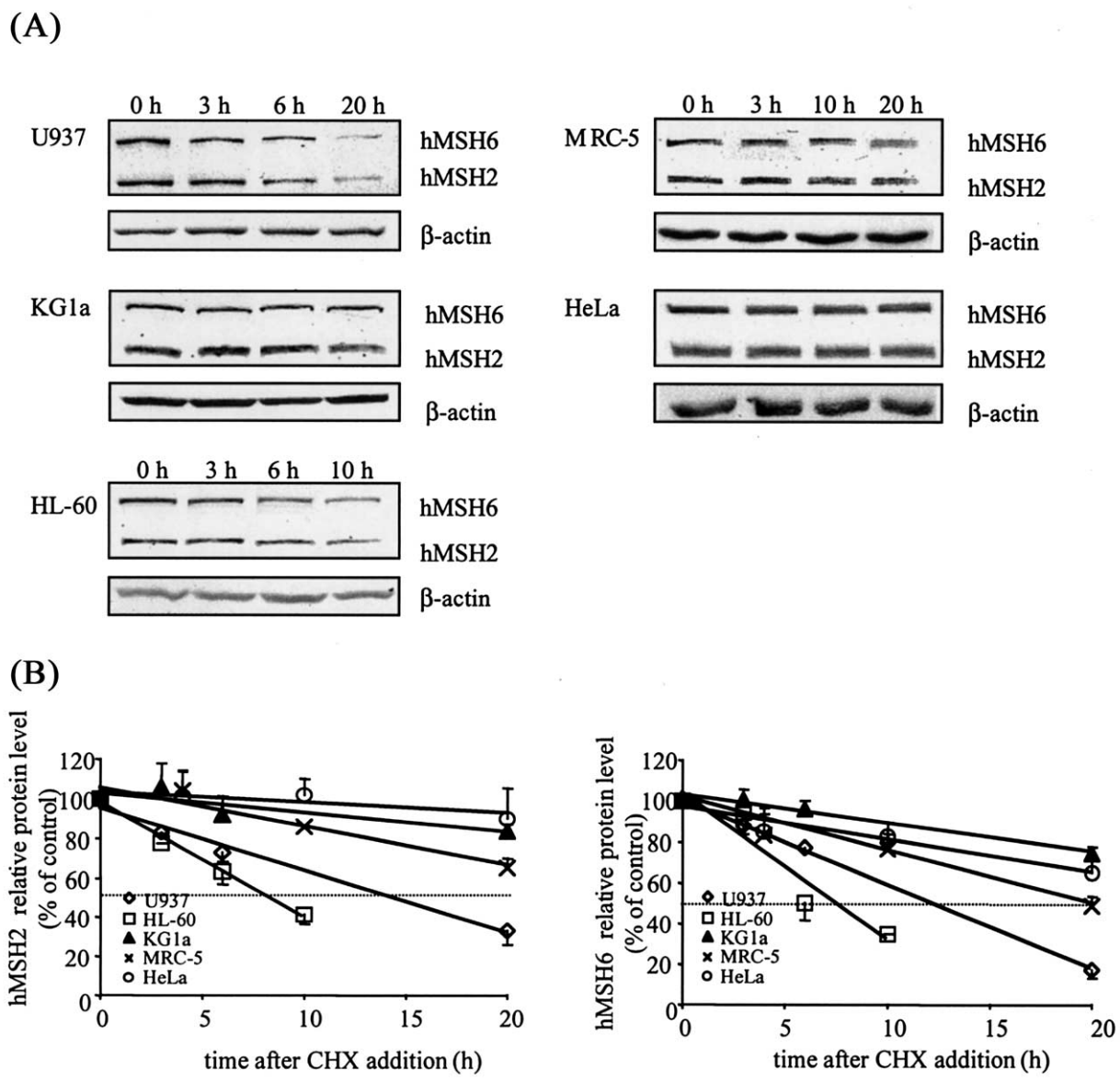

Fig. 2. A: hMSH2 and hMSH6 protein stability in human cell lines evaluated after cell incubation with 10 μg/ml CHX at the indicated times. Western blots of U937 and HL-60 cell extracts were overexposed in order to evaluate protein degradation. B: Densitometric analysis of hMSH2 and hMSH6 protein levels normalized to β-actin expression. Results are expressed as percentages of untreated control cells. Linear regression drawn in the graphs is calculated from the data. HL-60 cell exposure to CHX was limited to 10 h because of cytotoxic effects. Each data point is the mean (±S.D.) of three independent experiments.

3.3. *hMutSα proteins are degraded by the proteasome pathway*

In order to investigate the mechanisms of degradation of hMutSα proteins, we used different protease inhibitors. U937 cells were pretreated for 1 h with a caspase inhibitor (Z-VAD-FMK), serine protease inhibitors (dichloroisocoumarin (DCIC) or 1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK)), a calpain inhibitor (calpeptin), or proteasome inhibitors (MG132 or lactacystin), then treated with CHX for 20 h. In the absence of de novo protein synthesis, cell pretreatment with Z-VAD-FMK, DCIC, calpeptin, or TPCK did not block hMutSα protein degradation, whereas proteasome inhibitors MG132 and lactacystin fully inhibited hMutSα degradation (Fig. 3A). In all cell lines tested, MG132 was able to inhibit hMSH2 (Fig. 3A,B) and hMSH6 (Fig. 3A,C) protein degradation, even so the effect was less pronounced for the KG1a cells due to the naturally longer half-lives of the proteins in these cells. Moreover, treatment of U937 cells for 4 h with MG132 or lactacystin (Fig. 3D) increased hMSH2 and hMSH6 protein expression. Under our experimental conditions, treatment with proteasome inhibitors did not induce any cell death.

All these results strongly suggested that degradation of hMutSα proteins is dependent on the proteasome pathway.

3.4. *In vitro proteasome activity in human cell extracts*

Since the differences observed in hMutSα protein degradation could result from variations in the proteasome activity, we determined the overall proteasome activity in the different cell extracts by an in vitro assay. First, we checked that proteasome inhibitors completely blocked this activity (data not

shown). Unexpectedly overall proteasome activity varied with cell extracts but was lower in U937, HL-60 and HeLa than in KG1a and MRC-5 cell extracts (Fig. 4).

Therefore, these results precluded the hypothesis of a higher overall proteasome activity in U937 and HL-60 cell lines to explain the low hMutSα protein expression levels.

3.5. Ubiquitination of hMutSα proteins

Based on the data shown above, we speculated that the ubiquitination of hMutSα proteins prior to their degradation could differ in the different cell lines. Following immunoprecipitation with anti-Ub antibody, hMSH2 and hMSH6 proteins were recovered in the pool of ubiquitinated proteins in U937 and HL-60 cells while only faint ubiquitination of hMutSα was detected in KG1a, MRC-5 and HeLa cell lines (Fig. 5). As these proteins associate as a dimer, we can conclude that at least one of the partners exists in ubiquitinated form, more abundant in the U937 and HL60 cells than in KG1a, HeLa or MRC-5 cells.

These results suggested that ubiquitination may be a limiting step for hMutSα protein degradation in cells.

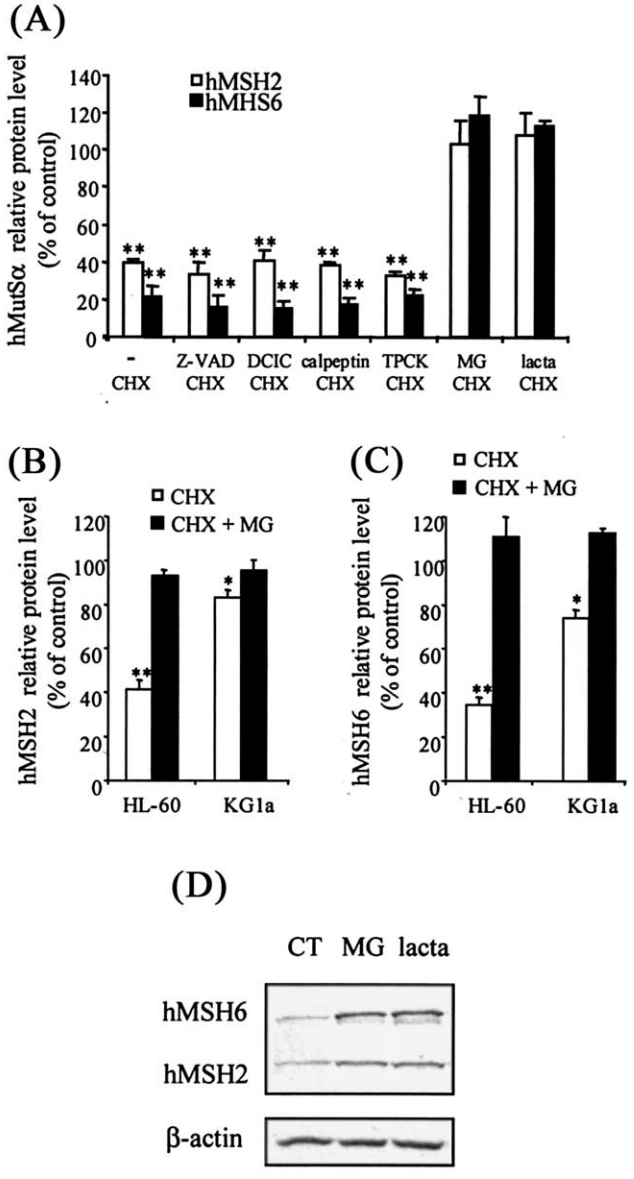

Fig. 3. Effect of different protease inhibitors on hMutSα protein stability in U937 cells (A), HL-60 and KG1a cell lines (B,C). When indicated, cells were pretreated for 1 h with 20 μM Z-VAD-FMK, 20 μM DCIC, 50 μg/ml calpeptin, 20 μM TPCK, 10 μM MG132 (MG), or 10 μM lactacystin (lacta). Cells were then incubated with 10 μg/ml CHX during 20 h. Densitometric analysis of hMSH2 and hMSH6 protein expression was performed. Results are expressed as percentages of untreated control cells after normalization to β-actin expression. Each data point is the mean (±S.D.) of three independent experiments. $*P<0.05$ and $**P<0.01$ versus untreated cells. D: Effect of proteasome inhibition on hMSH2 and hMSH6 protein expression. U937 cells were treated with MG132 (20 μM) or lactacystin (25 μM) for 4 h. β-Actin was used as loading control.

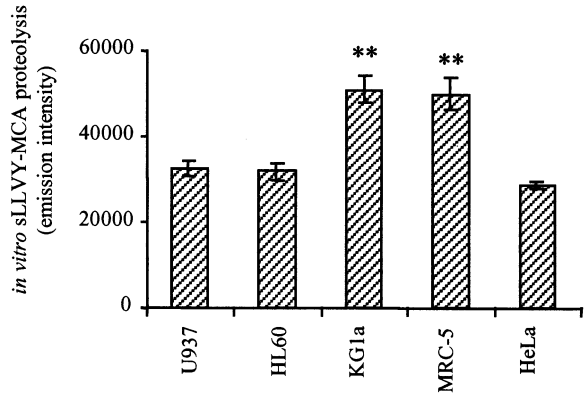

Fig. 4. Proteasome activity in human cell lines. Comparison of proteasome activity was done using sLLVY-MCA hydrolysis with cell extracts from different cell lines for 1 h at 37°C. Each data point is the mean of three independent experiments (±S.D.). $**P<0.01$ versus U937 cells.

3.6. In vitro degradation of hMutSα proteins by the Ub-dependent proteasome

In order to provide direct evidence that hMutSα proteins were degraded by the Ub-proteasome pathway, we measured the proteolysis of hMSH2 and hMSH6 proteins in an in vitro proteasome model from RRL. As shown in Fig. 6, hMutSα proteins from U937 cells were degraded only when both ATP and Ub were added. As expected, MG132 completely blocked

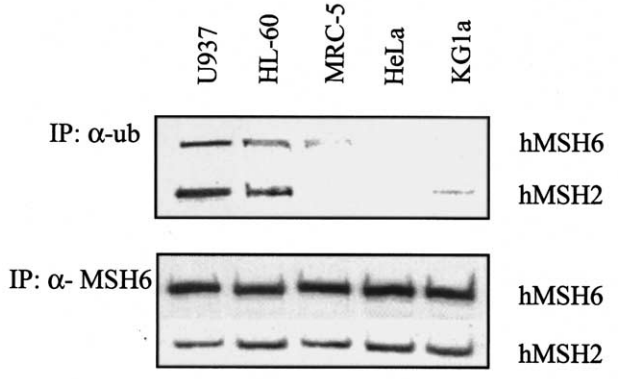

Fig. 5. Ubiquitination of hMutSα proteins in human cell lines. Immunoprecipitation was performed using anti-Ub and anti-MSH6 (as a control of the amount of hMutSα proteins) antibodies. Because of differences in basal hMutSα protein levels, immunoblots with anti-MSH2 and anti-MSH6 antibodies were performed with 1.2 mg of protein extracts from U937 and HL-60 cell lines and only 0.4 mg from KG1a, MRC-5 and HeLa cell lines.

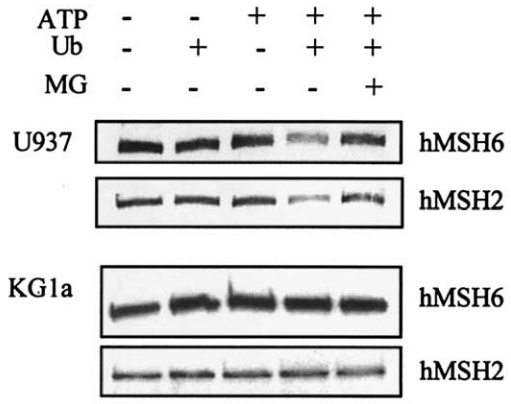

Fig. 6. Degradation of hMSH2 and hMSH6 proteins by the activation of Ub proteolysis in in vitro assay. Aliquots of immunoprecipitated hMSH6 and hMSH2 proteins from U937 and KG1a cell extracts were incubated with RRL. ATP, Ub and/or MG132 (MG) were added as indicated. hMutSα proteins are detected by immunoblots.

hMutSα protein degradation. These results confirmed that hMutSα proteins could be degraded in vitro by the Ub-proteasome pathway. In contrast, in the presence of both ATP and Ub, hMutSα proteins from KG1a cells were not degraded.

Altogether these results suggested that a defect in the Ub-proteasome pathway could not account for the lack of hMutSα protein ubiquitination and degradation observed in KG1a cells.

4. Discussion

This study shows for the first time that the Ub-proteasome complex is responsible for degradation of hMutSα proteins, and that this process is efficiently regulated in a cell-dependent manner. Therefore, proteasome-mediated degradation could play a major role in regulating hMutSα protein expression. Since hMSH2 and hMSH6 protein degradation rates are very similar, proteasome-mediated degradation could contribute to maintain a constant ratio between these two proteins.

The differences in protein degradation rates in the different human cell lines seemed specific for hMutSα proteins since other proteins including p21/Waf1, that are also degraded by the Ub-proteasome pathway [19], were degraded at the same rate in U937, HL-60, MRC-5 and HeLa cells (data not shown). These differences could result from different mechanisms. First, these cells may display different Ub-proteasome activities. However, using an in vitro assay, we found that overall proteasome activity did not correlate with hMutSα protein degradation rate in the different cell lines. Moreover, the degradation of hMutSα proteins immunoprecipitated from U937 and KG1a cells differed significantly when tested with the RRL system. These results argue against the implication of Ub-proteasome activity in the regulation of degradation rate. Second, the differences in expression of hMutSα proteins in U937 and KG1a cells could result from differences in their structure or conformation that would affect their ubiquitination and degradation. Specific protein–protein interaction or phosphorylation has been shown to influence structure or conformation of proteins, resulting in modulation of ubiquitination [20]. As far as hMutSα is concerned, the hypothesis of phosphorylation events is supported by the presence of putative phosphorylation sites in hMutSα proteins and by the recent demonstration that both hMSH2 and hMSH6 are substrates for various serine-threonine kinases [21]. These hypotheses are under current investigation.

Although proteasome inhibitors were able to increase hMutSα proteins, the recovery of hMutSα proteins was not accompanied by increased hMutSα activity (data not shown), suggesting that accumulated proteins were not active.

In cells displaying a high degradation rate of hMutSα proteins, we have already shown that low hMutSα protein expression level is a limiting factor for MMR activity [16]. Consequently, our present results suggest that the Ub-proteasome pathway may also influence MMR activity. The participation of the Ub-proteasome pathway in other DNA repair gene expression and activity has been reported already [13–15]. Therefore, the Ub-proteasome pathway plays a role as one of the regulators of DNA repair activities.

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