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Interaction of hHR23 with S5a

THE UBIQUITIN-LIKE DOMAIN OF hHR23 MEDIATES INTERACTION WITH S5a SUBUNIT OF 26 S PROTEASOME*

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hHR23B is one of two human homologs of the *Saccharomyces cerevisiae* nucleotide excision repair (NER) gene product RAD23 and a component of a protein complex that specifically complements the NER defect of xeroderma pigmentosum group C (XP-C) cell extracts *in vitro*. Although a small proportion of hHR23B is tightly complexed with the XP-C responsible gene product, XPC protein, a vast majority exists as an XPC-free form, indicating that hHR23B has additional functions other than NER *in vivo*. Here we demonstrate that the human NER factor hHR23B as well as another human homolog of RAD23, hHR23A, interact specifically with S5a, a subunit of the human 26 S proteasome using the yeast two-hybrid system. Furthermore, hHR23 proteins were detected with S5a at the position where 26 S proteasome sediments in glycerol gradient centrifugation of HeLa S100 extracts. Intriguingly, hHR23B showed the inhibitory effect on the degradation of ^{125}I-lysozyme in the rabbit reticulocyte lysate. hHR23 proteins thus appear to associate with 26 S proteasome *in vivo*. From co-precipitation experiments using several series of deletion mutants, we defined the domains in hHR23B and S5a that mediate this interaction. From these results, we propose that part of hHR23 proteins are involved in the proteolytic pathway in cells.

We have previously identified two distinct homologs of *Saccharomyces cerevisiae* nucleotide excision repair (NER)[1] factor RAD23, in human as well as in murine cells (1, 2). One of the human RAD23 homologs, designated hHR23B, was found to be tightly complexed with the XPC protein that plays an essential role in a subpathway of human NER operating genome-wide (1). Although a vast majority of the XPC protein is bound to hHR23B *in vivo*, another RAD23 homolog, hHR23A, is also capable of interacting with XPC at least *in vitro*. Using reconstituted cell-free NER reactions, we further showed that both hHR23 proteins enhance the repair activity of XPC, suggesting a possible functional interchangeability between the two RAD23 homologs (3).

Amino acid sequence comparison of the yeast RAD23 and its mammalian homologs revealed the existence of at least four distinct domains which are well conserved among these proteins (Ref. 2, see also upper part of Fig. 4*A*). First, this class of proteins is characterized by a Ub-like sequence at the amino terminus. It was genetically shown that this sequence is important for the biological function of yeast RAD23 (4). The second and fourth domains from the amino terminus are ubiquitin-associated domains (5), suggesting a possible involvement of the RAD23 as well as hHR23 proteins in certain pathways of ubiquitin metabolism (2). By deletion and truncation analysis of recombinant hHR23B protein, the third domain has been recently found to be responsible for binding the XPC protein (6). These deletion studies also revealed that the identified XPC-binding domain of hHR23B is necessary and largely sufficient for the hHR23B NER function *in vitro*: the Ub-like sequence and two copies of the ubiquitin-associated domains appear to be dispensable for the core part of NER. Therefore, it is conceivable that hHR23B, as well as other members of this class of proteins, may be associated with ubiquitin metabolic pathways outside the context of the core NER machinery. In agreement with this idea, hHR23B exists *in vivo* in a large excess over XPC (7, 8), suggesting it has extra functions without XPC.

To explore novel functions of the mammalian RAD23 homologs, it is of great interest to search for proteins other than XPC which interact with the hHR23 proteins. For this purpose, a yeast two-hybrid expression library was screened using hHR23B as a bait. Here we report the interaction of the hHR23 proteins with a 26 S proteasome subunit, S5a, indicating the association of the RAD23 homologs with the ubiquitin-dependent protein degradation machinery.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—The yeast two-hybrid screening was performed with the Matchmaker Two-Hybrid System (CLONTECH). The entire cDNA for hHR23B was fused in-frame with the GAL4 DNA-binding domain in pGBT9 vector (9). The resulting plasmid, pGBT-hHR23B, was used for transformation of a yeast strain, HF7c,

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[1] The abbreviations used are: NER, nucleotide excision repair; hHR23, human homolog of RAD23; Ub, ubiquitin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; 6His, hexahistidine; GST, glutathione *S*-transferase.

and a Trp^+ clone was selected. A human cDNA library for the two-hybrid screening was constructed with TimeSaver cDNA synthesis kit and Directional cloning toolbox (both from Amersham Pharmacia Biotech). Complementary DNAs were synthesized with *Not*I/oligo(dT) primers and poly(A)$^+$ RNA from an SV40-transformed fibroblast cell line, WI38VA13. After the second strand synthesis, the cDNAs were ligated to *Eco*RI adaptors and then digested with *Not*I, resulting in double-stranded cDNA fragments bearing *Eco*RI and *Not*I overhangs on the upstream and downstream ends, respectively. The cDNAs were subsequently cloned into pGAD424 vector encoding the GAL4 activation domain (9), and introduced into *Escherichia coli*, DH5α. The plasmid DNA was isolated from 7.8×10^7 *E. coli* colonies. The yeast strain bearing pGBT-hHR23B was further transformed with the cDNA library, and positive clones were selected by histidine prototrophs. The His^+ clones were further verified for β-galactosidase activity using a filter lift assay (10).

Glycerol Gradient Sedimentation Analysis—Cytoplasmic S100 fraction was prepared from HeLa cells as described previously (11). The HeLa S100 (7 mg of protein) was supplemented with ATP to a final concentration of 2 mM, and incubated on ice for 1 h. The extract was overlaid onto a 10–40% glycerol gradient (30 ml) in 25 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 2 mM ATP, and subsequently centrifuged at 22,500 rpm (83,000 × *g*) for 22 h at 4 °C. One-milliliter fractions were collected from the bottom of the tube. The proteasome activity of each fraction (150 μl) was assayed as described previously (12).

Immunoprecipitation—Each of anti-p45 monoclonal antibody and anti-ubiquitin monoclonal antibody (2C5; MBL Co. Ltd., Japan), were mixed with Protein G-Sepharose (Amersham Pharmacia Biotech), respectively, in buffer C (20 mM Tris-HCl (pH 7.9), 200 μM EDTA, 20% glycerol, 0.1 M KCl, 2 mM ATP, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 0.25 mM PMSF, 10 μM 2-mercaptoethanol) containing 200 μg/ml bovine serum albumin at 4 °C for 30 min with rotation. The beads were then washed three times with buffer C. The antibody bound-Sepharose beads (5 μl) were incubated with 150 μg of whole cell extract prepared from HeLa cells (13) in 80 μl of buffer C at 4 °C for 1 h with rotation. The mixture was centrifuged, and the precipitates were washed three times with buffer C. The bound proteins were extracted by boiling in SDS sample buffer, separated by 8% SDS-PAGE, and analyzed by immunoblotting with anti-hHR23B and anti-XPC antibody.

Preparation of Recombinant hHR23, S5a, and NEDD8 Proteins—Recombinant hHR23A and hHR23B proteins (designated as rhHR23A and rhHR23B) as well as a series of deletion mutants of hHR23B were expressed in *E. coli* and purified as described previously (6). Ub-rhHR23B, which has the ubiquitin sequence instead of the original Ub-like sequence on its NH_2 terminus, was constructed. Ubiquitin cDNA was obtained from a human cDNA library using polymerase chain reaction technique, and inserted in-frame into pET-24d (Novagen) which harbors hHR23B with an NH_2-terminal deletion encoding amino acids 73–409 (6). The resulting plasmid expresses hHR23B with the ubiquitin sequence on its NH_2 terminus instead of the Ub-like domain in *E. coli*. The expression and purification of the protein was performed as described previously (6). Recombinant human S5a (designated as rS5a) protein was expressed in *E. coli* BL21(DE3) using pET3a vector (Novagen). Cells containing the S5a construct were grown at 30 °C, induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h, collected by centrifugation of approximately 1 liter of culture, washed with 50 mM Tris-HCl (pH 7.5) containing 10% glycerol, and suspended in 20 mM sodium phosphate (pH 6.8), 0.3 M NaCl, 0.25 mM PMSF. The suspension was incubated on ice for 15 min with 1 mg of lysozyme/ml, frozen in liquid nitrogen, and thawed at 4 °C. The lysate was adjusted to 35% saturation of ammonium sulfate (0.164 g of solid/ml). The precipitates were collected by centrifugation (20,000 × *g*, 30 min), dissolved in 20 mM sodium phosphate (pH 6.8) containing 0.25 mM PMSF and then dialyzed against 20 mM sodium phosphate (pH 6.8) containing 0.8 M ammonium sulfate. After removal of insoluble materials by centrifugation, the dialysate was loaded on a 1-ml butyl-Sepharose 4FF column (Amersham Pharmacia Biotech) equilibrated with 20 mM sodium phosphate (pH 6.8) containing 0.8 M ammonium sulfate. The column was washed with the same buffer and bound proteins were eluted with 40 ml of a decreasing ammonium sulfate gradient from 0.8 to 0 M. rS5a, detected by SDS-PAGE and Coomassie Brilliant Blue staining, was eluted during the last part of the gradient. The fractions containing rS5a were pooled. After the dialysis against 20 mM sodium phosphate (pH 6.8) containing 0.25 mM PMSF, the dialysate was loaded on a 1-ml Hi-Trap Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated with the same buffer. After the column was washed with an excess amount of the same buffer, bound proteins were eluted with 20 mM sodium phosphate (pH 6.8), 0.25 mM PMSF, 1 M NaCl. The peak fractions of rS5a were stored at −80 °C. S5a was also expressed as 6His- or GST-tagged versions using 6HisT-pET11d or pGEX-2T(+) vector, respectively. The tagged rS5a proteins were purified by nickel-chelating Sepharose (for 6His-tagged proteins) or glutathione-Sepharose (Amersham Pharmacia Biotech) column chromatography (for GST-tagged proteins). For generation of various types of S5a deletion mutants, 5′ primers were designed with *Nde*I restriction sites at selected internal methionine codons. 3′ Primers containing *Eco*RI restriction sites were also designed to generate COOH-terminal deletion mutants. The polymerase chain reaction products were inserted into 6HisT-pET11d vector for expression. A series of deletion mutants of S5a was expressed and purified as described above. NEDD8 (14) was expressed as 6His-tagged version using pET24a vector. The tagged NEDD8 (rNEDD8–6His) protein was purified as described above.

Protein Degradation Assay—Lysozyme was iodinated with $Na^{125}I$ using Iodo-Beads (Pierce) according to the manufacture's instruction. Assay of ^{125}I-lysozyme degradation was performed by the procedure described previously (15) with the following modifications. Briefly, 40 μl of the rabbit reticulocyte extracts were incubated for 30 min at 37 °C in a total volume of 100 μl which contained 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, and either an ATP-regenerating system (2 mM ATP, 10 μg/ml creatin phosphokinase, 10 mM phosphocreatin) or ATP-depleting system (1 μg/ml hexokinase, 10 mM glucose) prior to adding ^{125}I-lysozyme (20,000 cpm). After incubation for 1 h at 37 °C, the reaction was terminated by addition of 575 μl of 10% trichloroacetic acid with 125 μl of 4% of bovine serum albumin as a carrier. Radioactivity in the supernatants and the precipitated pellets were counted by γ spectrophotometry (WALLAC, WIZARD 1840). To assess the effect of S5a or hHR23B on the ^{125}I-lysozyme degradation, each recombinant proteins were incubated with reticulocyte extracts for 30 min on ice before the addition of ^{125}I-lysozyme. In experiments designed to demonstrate the stability of rhHR23B-6His, rhHR23B-6His was incubated at 37 °C for 1 h in reticulocyte extracts and collected by nickel-chelating Sepharose beads as described above. Then, the collected proteins were subjected to 12% SDS-PAGE, and detected by monoclonal antibody raised against pentahistidine (QIAGEN).

In Vitro Binding Assay—The rhHR23B-6His (full-length, truncated, or Ub-fused) proteins (30 pmol) or the rNEDD8–6His protein (30 pmol) were incubated with non-tagged rS5a protein (3 pmol) in buffer H (20 mM HEPES (pH 7.9), 0.1 M NaCl, 20% glycerol, 0.1% Nonidet P-40, 200 μg/ml bovine serum albumin, 0.25 mM PMSF, 10 μM 2-mercaptoethanol) on ice for 1 h. Each protein sample was mixed with a 2-fold volume of a suspension of nickel-chelating Sepharose beads in buffer H containing 20 mM imidazole, and incubated for 1 h at 4 °C. The mixture was centrifuged, and the beads were washed three times with buffer H containing 60 mM imidazole. The bound proteins were extracted by boiling in SDS sample buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting with anti-S5a antibody. The *in vitro* binding assay for a series of 6His-tagged rS5a mutants (30 pmol) with either non-tagged rhHR23A or rhHR23B (3 pmol) was performed as described above except that either anti-hHR23A or anti-hHR23B antibody was used for immunoblotting.

Competition Assay—The rhHR23B-6His protein (3 pmol) was mixed with various amounts of competitor in 100 μl of buffer H, and then incubated with GST-rS5a (15 pmol) on ice for 1 h. Both the rhHR23B-(1–87) protein (containing the NH_2-terminal Ub-like portion of hHR23B) and the ubiquitin molecule were used as competitor. Then glutathione-Sepharose beads were added and mixed at 4 °C overnight. The mixture was centrifuged, and the resin was washed with buffer H. The bound proteins were extracted by boiling in SDS sample buffer, separated by 10% SDS-PAGE, and analyzed by immunoblotting with anti-hHR23B antibody.

Antibodies—Antibodies against hHR23B, hHR23A, and XPC were obtained as described previously (7). Anti-S5a antibody was obtained by immunization of rabbits with a 6His-tagged rS5a, and purified with affinity chromatography at MBL Co. Ltd. The serum of anti-p45 monoclonal antibody was kindly provided by Klavs B. Hendil.

Other Methods—SDS-PAGE was performed as described by Laemmli (16). For the immunoblotting, proteins separated on SDS gels were electrotransferred onto polyvinylidene difluoride membrane (Immobilon-P; Millipore), at 8 V/cm overnight in ice-cold transfer buffer (50 mM Tris, 38.4 mM glycine, 0.01% SDS, 15% methanol). The membranes were successively incubated in blocking buffer (5% skim milk in 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% Tween 20), with first antibody in blocking buffer, and then with anti-rabbit or anti-mouse $F(ab')_2$ antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech) in blocking buffer. Detection was carried out with SuperSignal Substrate (Pierce) according to the manufactures instructions. Protein

TABLE I
Protein-protein interactions assessed by the yeast two-hybrid system
Yeast strain, HF7c, was transformed with the pGBT9 bait and pGAD424 target expression vectors.

	Bait construct	Target construct	Histidine prototroph[a]	β-Galactosidase assay[a]
1.	pGBT9 only	pGAD424 only	−	−
2.	pGBT9-hHR23B	pGAD424 only	−	−
3.	pGBT9 only	pGAD424-XPC	−	−
4.	pGBT9-hHR23B	pGAD424-XPC	+	+
5.	pGBT9 only	pGAD424-S5a	−	ND[b]
6.	pGBT9-hHR23B	pGAD424-S5a	+	+
7.	pGBT9-hHR23B	pGAD424-S5a114–377	+	++
8.	pGBT9-hHR23B	pGAD424-S5a184–377	+	++
9.	pGBT9-hHR23B	pGAD424-S5a192–377	+	++
10.	pGBT9-hHR23A	pGAD424 only	−	ND
11.	pGBT9-hHR23A	pGAD424-S5a	+	ND

[a] −, no evidence for histidine prototrophy and color change of the yeast colony; + and/or ++, histidine prototrophy or intensity color.
[b] ND, not determined.

concentration was measured according to the method of Bradford (17), using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories) and bovine serum albumin as a standard.

RESULTS

hHR23B Interacts with S5a, a Component of the Human 26 S Proteasome Regulatory Subunit—To isolate proteins which interact with hHR23B, we performed a yeast two-hybrid screening. The interaction of the hHR23B fusion protein with its known partner, XPC, was confirmed both by histidine prototrophy conferred on the yeast strain and by an intense blue color upon *in situ* β-galactosidase assay (Table I, line 4). These results indicate that hHR23B was expressed and interacted specifically with a known natural target molecule in yeast as reported previously (18). The screening of 1.5×10^7 yeast transformants yielded 20 candidate His^+ and $LacZ^+$ clones, 17 of which contain cDNA encoding the human S5a protein, one of the regulatory subunits of 26 S proteasome (19). One clone covers full-length of the S5a cDNA, the others encode S5a with NH_2-terminal truncation of various size, suggesting that the COOH-terminal part of S5a may be responsible for interaction with hHR23B. To confirm the specificity of the observed interaction, pGAD plasmids were isolated from several clones, and reintroduced into yeast in combination of pGBT-hHR23B. As expected, the histidine prototrophy and β-galactosidase activity were observed in yeast expressing both S5a protein and hHR23B (Table I, lines 6–9). It should be noted that the interaction of truncated S5a with hHR23B was stronger (lines 7–9) than that of intact S5a (line 6) as determined in the β-galactosidase assay (see "Discussion"). Furthermore, another human homolog of RAD23, hHR23A, was also found to interact with S5a (Table I, line 11). S5a is known as a multiubiquitin chain-binding protein. Since both hHR23 proteins have highly conserved Ub-related sequences on NH_2 termini (1, 2), we speculated that these sequences are involved in the interaction with S5a.

hHR23B Interacts with 26 S Proteasome in Cell Extracts—The above results of a yeast two-hybrid screening showed that hHR23 proteins interact with S5a, suggesting that hHR23 proteins might be able to associate with 26 S proteasome via the interaction with S5a *in vivo*. Such an association can be analyzed by immunoblot analysis of fractions of cell extracts separated through glycerol gradient centrifugation after preincubation of the extracts in the presence of 2 mM ATP, which promotes assembly of 26 S proteasome in cell extracts (20). Thus, identification of putative subunits of the proteasome can be determined by analysis of their distribution over the gradient. When the distribution of hHR23B was analyzed, the protein was found to migrate with S5a peaking at fraction 9 (Fig. 1*A*), although the vast majority of hHR23B was found in slower sedimenting fractions (Fig. 1*A*). Under these conditions, most

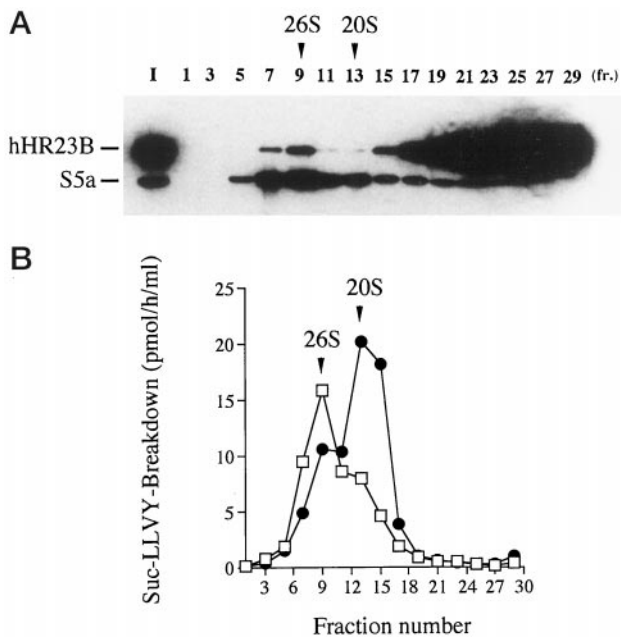

FIG. 1. **Co-fractionation of hHR23 proteins with the 26 S proteasome through glycerol density sedimentation.** HeLa S100 was sedimented on a 10–40% glycerol density gradient by centrifugation in the presence of 2 mM ATP, and fractions were collected. *A*, the distribution of hHR23B and S5a was assessed by immunoblotting. Proteins (present in 150 μl of each fraction) were precipitated with cold acetone and subjected to immunoblot analysis. *fr.*, fraction; *I*, 10% input. *B*, Measurement of the proteasome activity. S values are indicated with *arrowhead*. The *open squares* represent the proteasome activity in the absence of 0.02% SDS. *Closed circles* represent the proteasome activity in the presence of 0.02% SDS. *Numbers* at the *bottom* of *panel B* correspond to the fraction numbers of *panel A*.

of the 26 S proteasome activity was observed in fraction 9 (Fig. 1*B*). Similar results were obtained in the case of hHR23A experiments (data not shown). We previously reported that the sedimentation coefficient of the XPC-hHR23B complex is 6.2 S (1). Furthermore, in the absence of ATP, hHR23 proteins were sedimented with 19 S regulatory complex through glycerol gradient centrifugation (data not shown). To explore the association between hHR23 proteins and 26 S proteasome in a different way, HeLa whole cell extracts were used for immunoprecipitation with monoclonal antibody raised against p45, one of the regulatory components of 26 S proteasome (21). Fig. 2 indicates that a part of hHR23B associates with the proteasome regulatory complex containing S5a in the cell extracts, although co-precipitation of XPC was undetected. These results strengthened our hypothesis that hHR23 proteins are associated with 26 S proteasome at least through the interaction with S5a in cell extracts.

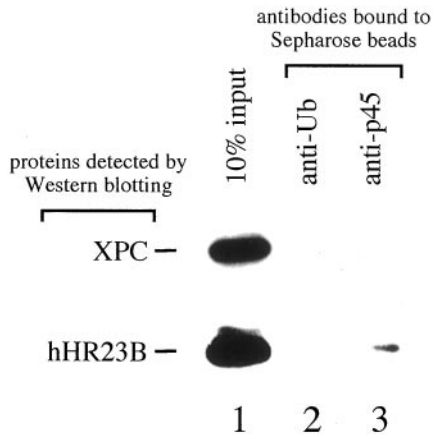

FIG. 2. **Co-immunoprecipitation of 26 S proteasome and hHR23B from HeLa whole cell extract.** Antibody bound-Sepharose beads were incubated with HeLa whole cell extract in buffer C at 4 °C for 1 h with rotation. Bound proteins were separated by 8% SDS-PAGE. 10% input of HeLa whole cell extract is shown in *lane 1*. Anti-ubiquitin antibody (*anti-Ub*) served as a negative control (*lane 2*). To precipitate the proteasome complex, anti-p45 antibody (*anti-p45*) was used (*lane 3*). XPC, a known partner of hHR23B, and hHR23B were assessed by immunoblotting using anti-XPC or anti-hHR23B antibodies, respectively.

Inhibition of Lysozyme Degradation by hHR23B—To explore the physiological meaning of the interaction between hHR23 proteins and 26 S proteasome, the effects of hHR23B on ^{125}I-lysozyme degradation was demonstrated using rabbit reticulocyte extracts. It was previously reported that the *Arabidopsis* S5a inhibits the degradation of multiubiquitinated lysozyme in reticulocyte extract (22). As shown in Fig. 3*A*, human S5a was sufficient to interrupt ^{125}I-lysozyme degradation in reticulocyte extracts as well (*lane 2*). Intriguingly, ^{125}I-lysozyme degradation was also inhibited by the addition of rhHR23B-6His in a dose-dependent manner (*lanes 3–6*). It is possible that rhHR23B-6His works as a competitor for ^{125}I-lysozyme degradation by proteasome in reticulocyte extracts, because hHR23 proteins are associated with the S5a subunit of 26 S proteasome as described above. Surprisingly, no proteolysis of rhHR23B-6His was observed during a 3-h incubation (Fig. 3*B*). These data indicate that the physiological relation lies between hHR23B and 26 S proteasome. It has been generally accepted that protein substrate for the ubiquitin-dependent proteolysis would be multiubiquitinated by ubiquitination and degraded by 26 S proteasome in rabbit reticulocyte extracts. It is likely, therefore, that the Ub-like domain of the hHR23 proteins is very competitive with multiubiquitin chain.

Determination of the S5a-binding Domain in hHR23B—To further explore the meaning of the interaction between hHR23 proteins and S5a, several experiments were performed using recombinant S5a (rS5a) expressed in *E. coli*. We previously identified the region in hHR23B that affects the interaction with XPC using 6His-tagged recombinant hHR23B (rhHR23B-6His) proteins with various deletion mutant (6). These mutant proteins were used to identify the region responsible for the interaction with S5a. Mutant rhHR23B-6His proteins, summarized in Fig. 4*A*, were adsorbed to nickel-chelating Sepharose beads, and then incubated with non-tagged rS5a protein. Co-precipitation of S5a was assessed by immunoblotting. As shown in Fig. 4*B*, rhHR23B lacking 72 amino acids from the NH_2 terminus failed to interact with S5a (*lane 3*), whereas the polypeptide corresponding to only 87 amino acids from the NH_2 terminus of hHR23B, designated as rhHR23B-(1–87), was sufficient to precipitate the S5a molecule (*lane 12*). As we reported previously, the NH_2-terminal region (amino acids 1 to 79) in

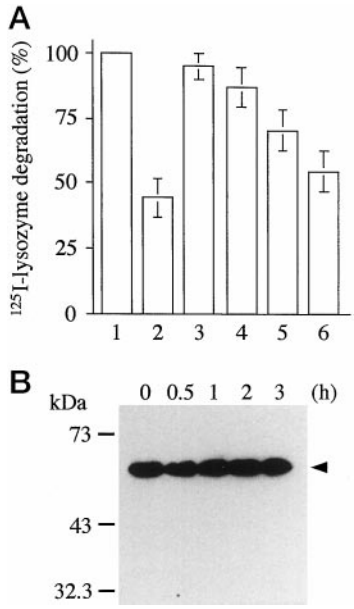

FIG. 3. **Inhibition of ^{125}I-lysozyme degradation by rhHR23B-6His.** *A*, ^{125}I-lysozyme was incubated with rabbit reticulocyte extracts at 37 °C for 1 h. Proteolysis of ^{125}I-lysozyme was measured in the presence of either rhS5a or rhHR23B-6His. *Lane 1* shows the ATP-dependent ^{125}I-lysozyme degradation. Relative degradation of ^{125}I-lysozyme in the presence of rhS5a (20 μg) was shown in *lane 2*, and in the presence of rhHR23B-6His (1, 5, 20, and 30 μg) was shown in *lanes 3–6*, respectively. Each value was the mean ± S.D. of at least three independent experiments. *B*, the stability of rhHR23B-6His (20 μg) in the rabbit reticulocyte lysate was assessed. After incubation at 37 °C for the indicated time above each lane, the materials bound to the nickel-chelating Sepharose beads were recovered. The presence of precipitated hexahistidine-tagged protein was detected by immunoblotting using anti-pentahistidine antibody. *Closed arrowhead* indicates the intact rhHR23B-6His protein. Molecular weight was indicated on the *left side* of the panel.

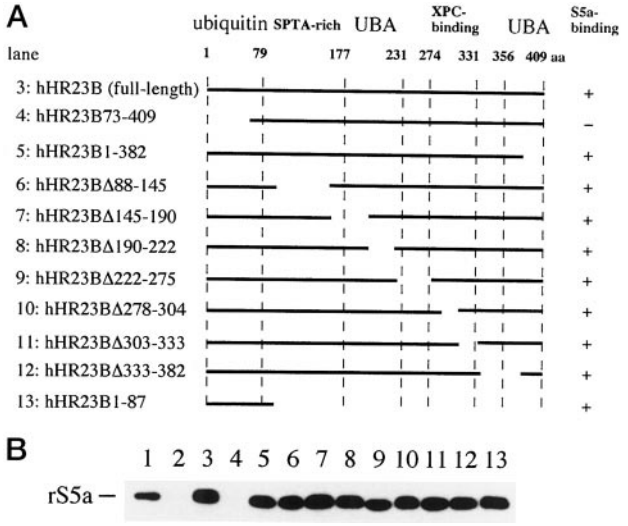

FIG. 4. **S5a binding activities of truncated rhHR23B-6His proteins.** *A*, summary of the mutant 6His-tagged rhHR23B (rhHR23B-6His) proteins. *Ubiquitin*, ubiquitin-like region; *SPTA-rich*, four kinds of related amino acids (S, P, T, and A) are predominant in this region; *UBA*, ubiquitin-associated domain; *XPC-binding*, XPC-binding domain; *aa*, amino acids. *B*, the presence of precipitated rS5a was assessed by immunoblotting. 10% input of rS5a is shown in *lane 1*. The proteins bound to the nickel-chelating Sepharose beads are shown in *lanes 2–13*. *Lane 2* is a negative control (no hHR23B protein) and *lanes 3–13* correspond to lane numbers in *panel A*.

hHR23B shares a homology to ubiquitin (1). Identification of the Ub-like sequence as the S5a-interacting domain of hHR23B raised the possibility that the S5a protein binds to any proteins

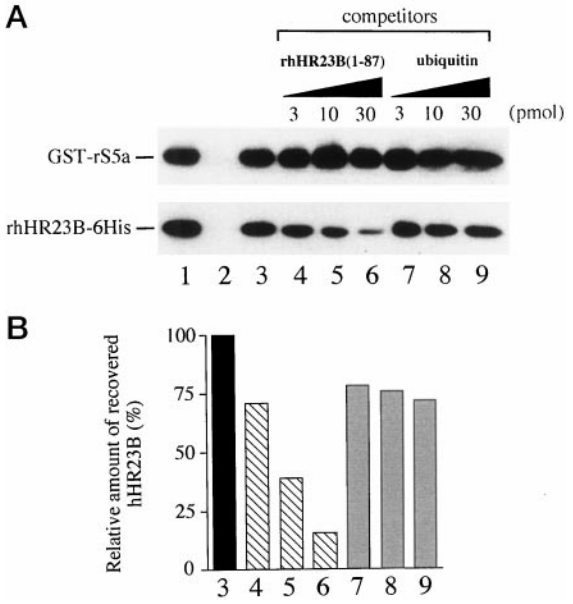

FIG. 5. **Effects of hHR23B-(1–87) and ubiquitin on the interaction between rhHR23B and rS5a.** *A*, rhHR23B-6His (3 pmol) and GST-rS5a (15 pmol) were mixed, and the materials bound to glutathione-Sepharose beads were recovered as described under "Experimental Procedures." Bound proteins to glutathione-Sepharose beads are shown in *lanes 2–9*. 50% input of GST-rS5a and 20% input of rhHR23B-6His are shown in *lane 1*. Either rhHR23B-(1–87) or ubiquitin was used as a competitor with the indicated amount above each lane. *B*, graphical representation of the results of *panel A*, presented as the percentage of recovered rhHR23B-6His without competitor. The signals of *panel A* were quantified using NIH Image software. For proper comparison, the quantified amount of hHR23B was normalized to the amount of the recovered S5a. The *solid bar* represents the recovered hHR23B without any competitor. The *striped bars* represent the amount of hHR23B in the presence of hHR23B-(1–87). The *gray bars* represent the amount of hHR23B in the presence of ubiquitin. The *numbers* at the *bottom* correspond to the *lane numbers* in *panel A*.

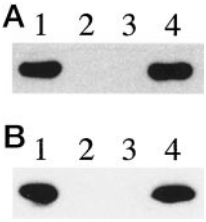

FIG. 6. **S5a binding activity of Ub-rhHR23B-6His.** rS5a was mixed with either Ub-rhHR23B-6His or rhHR23B-6His in buffer H at 4 °C for 1 h, and the materials bound to the nickel-chelating Sepharose beads were recovered. Bound proteins to the nickel-chelating Sepharose beads without protein (*lane 2*), with Ub-rhHR23B-6His (*panel A, lane 3*) or rNEDD8–6His (*panel B, lane 3*), and with rhHR23B-6His (*lane 4*) are shown. *A* and *B*, 20% input of rS5a is shown in *lane 1*. The presence of precipitated rS5a was assessed by immunoblotting using anti-S5a antibody.

which are conjugated with ubiquitin. To examine whether this is the case, we performed competition experiments using either free ubiquitin itself or the NH_2-terminal Ub-like domain of hHR23B. As shown in Fig. 5, hHR23B was detected in the precipitate fraction with GST-rS5a and glutathione-Sepharose beads (*lane 3*). Interestingly, the amount of precipitated hHR23B by GST-rS5a was dramatically decreased by the addition of rhHR23B-(1–87) in a concentration-dependent manner (Fig. 5, *lanes 4–6*), while the same concentration of ubiquitin monomer only slightly affected the interaction between GST-rS5a and rhHR23B (Fig. 5, *lanes 7–9*). One might argue that the S5a protein efficiently binds both Ub-like domains and ubiquitin itself only when fused to other proteins, via the COOH terminus, because the hHR23B-(1–87) has extra 8 amino acids beyond the Ub-like domain. To test this hypothesis, we prepared a chimeric protein, in which the Ub-like in hHR23B was replaced by ubiquitin itself (Ub-rhHR23B-6His). S5a was incubated with either rhHR23B-6His or Ub-rhHR23B-6His and pulled down with nickel-chelating Sepharose beads. As shown in Fig. 6*A*, S5a was hardly co-precipitated with Ub-rhHR23B-6His (*lane 3*), while rhHR23B-6His efficiently binds S5a (*lane 4*). To further confirm the specificity of the interaction between hHR23B and S5a, another binding experiment was performed using one of the Ub-like proteins, NEDD8 (14). NEDD8 shows high homology to ubiquitin (81 amino acids which show 58% identity and 79% similarity with ubiquitin), but its role in proteolysis has not been identified. Fig. 6*B* shows the specific interaction between hHR23B and S5a (*lane 4*) but not between NEDD8 and S5a (*lane 3*). These results indicate that the Ub-like domain of hHR23B is necessary and sufficient to interact with S5a, and establish the specificity of S5a for binding to the Ub-like sequence.

Determination of the hHR23-binding Domain in S5a—To localize the region in S5a that mediates the interaction with both hHR23 proteins, a series of truncated S5a proteins was also prepared (Fig. 7*A*). Amino acid sequence comparison among the S5a homologs in eukaryotes revealed a highly conserved region in the COOH-terminal half, which contains some hydrophobic amino acids (23). To examine binding to both hHR23 proteins, the 6His-tagged rS5a (full-length or truncated) proteins were incubated with either non-tagged rhHR23B or rhHR23A. The tagged proteins were pulled down with nickel-chelating Sepharose beads, and the presence of both rhHR23 proteins in the precipitate fractions was assessed by immunoblotting. As shown in Fig. 7, the rhHR23 proteins itself did not bind nickel-chelating Sepharose beads (Fig. 7, *panels B* and *C*, *lane 2*). In the presence of the full-length 6His-tagged rS5a, detectable amounts of both rhHR23 proteins were precipitated (Fig. 7, *panels B* and *C*, *lane 3*), indicating that not only hHR23B but also hHR23A formed a physical complex with rS5a *in vitro* as expected from the two-hybrid assay. When no more than 262 amino acids were deleted from the NH_2 terminus, both hHR23 proteins were still bound to the mutant rS5a proteins (Fig. 7, *panels B* and *C*, *lanes 4–6*). As for the deletion from the COOH terminus, both hHR23 proteins were detected in the bound fractions with the 6His-tagged rS5a lacking amino acids from residue Gly^{344} onwards to the COOH terminus (Fig. 7, *panels B* and *C*, *lane 7*). However, further deletion toward the NH_2 terminus abolished the hHR23 binding activities almost completely (Fig. 7, *panels B* and *C*, *lanes 8* and *9*). These results indicate that the hHR23-binding domain is located within the region covering amino acids Met^{263} to Pro^{343} of S5a (Fig. 8*A*). To further define the region for hHR23-binding in S5a, another series of mutant S5a proteins were subjected to the binding assay (Fig. 8*A*). Fig. 8 (*panels B* and *C*) shows that polypeptide corresponding to amino acid 263–307 of S5a efficiently binds both hHR23B and hHR23A (*lane 6*). Moreover, amino acids from Met^{263} to Met^{281} are necessary for the binding (Fig. 8, *B* and *C*, *lanes 3* and *4*).

DISCUSSION

RAD23 was originally isolated in a screening for ultraviolet-sensitive mutants (24) and was the first Ub-like protein identified in yeast (4). The Ub-like sequence of RAD23 was shown to be important for its NER function in yeast. However, a requirement for the Ub-like sequence in hHR23B is not clear at least in the cell-free NER system (6). Previously, we have examined the interaction of hHR23B and XPC proteins by using several deletion mutants of hHR23B protein. The XPC-binding domain was mapped near the COOH terminus of hHR23B, to a region

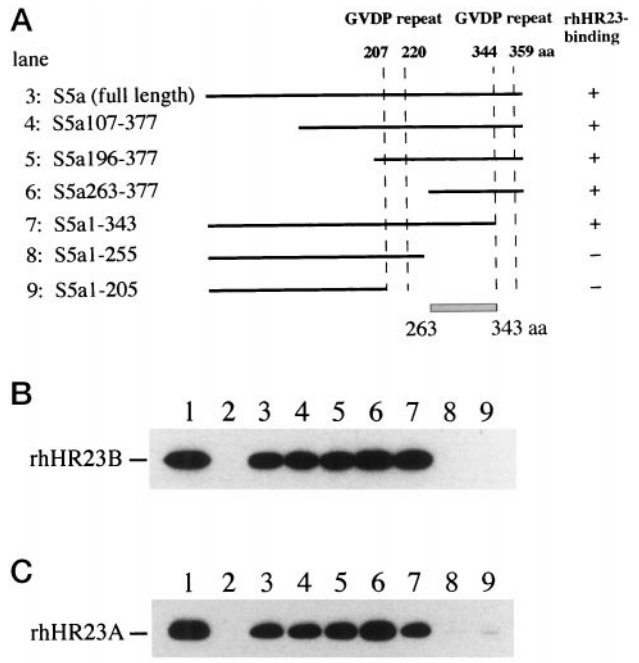

FIG. 7. **Binding of rhHR23 proteins to truncated 6His-tagged rS5a.** *A*, a summary of the mutant 6His-tagged rS5a proteins. The *gray bar* represents the binding domain for the hHR23 proteins. *GVDP repeat*, region which includes the GVDP tetrapeptide highly conserved among S5a homologs; *aa*, amino acids. *B* and *C*, the presence of precipitated rhHR23B (*panel B*) and rhHR23A (*panel C*) was assessed by immunoblotting. 20% input of rhHR23B (*panel B*) and rhHR23A (*panel C*) is shown in each *lane 1*. The proteins bound to the nickel-chelating Sepharose beads are shown in *lanes 2–9*. *Lane 2* is a negative control (no S5a protein), and *lanes 3–9* correspond to *lane numbers* in *panel A*.

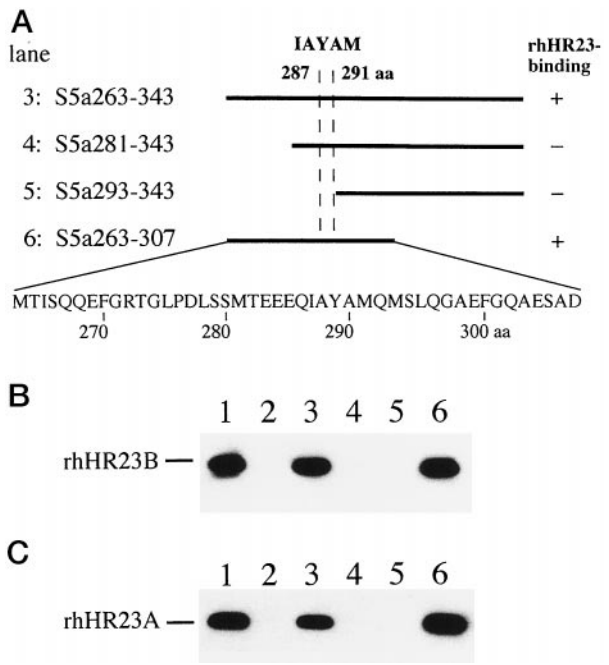

FIG. 8. **Further analysis of the S5a region binding to the hHR23 proteins.** *A*, summary of the mutant 6His-tagged rS5a proteins. The amino acid sequence of S5a263–307 is presented in the one-letter amino acid code. *IAYAM*, five hydrophobic amino acids which are highly conserved in S5a homologs; *aa*, amino acids. *B* and *C*, the presence of precipitated rhHR23B (*panel B*) and rhHR23A (*panel C*) was assessed by immunoblotting. 20% input of rhHR23B (*panel B*) and rhHR23A (*panel C*) is shown in each *lane 1*. The proteins bound to the nickel-chelating Sepharose beads are shown in *lanes 2–6*. *Lane 2* is a negative control (no S5a protein) and *lanes 3–6* correspond to *lane numbers* in *panel A*.

with a putative amphipathic helical character but the NH_2-terminal Ub-like sequence was not absolutely required for the interaction. Therefore, it is unlikely that the Ub-like sequence plays a crucial role in complex formation between XPC and hHR23B. Furthermore, the majority of hHR23 proteins are free from XPC in human cells (7, 8). These results strongly suggest that the hHR23 proteins have an additional role in cells other than the complex formation with XPC.

Physiological Interaction between hHR23B and 26 S Proteasome—Here we described the physical association between hHR23 proteins and S5a, one of the regulatory subunits of 26 S proteasome, using a yeast two-hybrid system. Furthermore, we report here that hHR23B associates with 26 S proteasome by both the glycerol gradient sedimentation and co-immunoprecipitation (Figs. 1 and 2). As shown in the yeast two-hybrid experiment, the interaction of NH_2-terminal-truncated S5a with hHR23B was stronger than that of intact S5a (Table, lines 6–9). This finding can be explained in two ways. One is that the NH_2-terminal portion of S5a reduces the interaction of S5a with hHR23B. The other is that the deletion mutants have a better conformation to interact with hHR23B than the full-length S5a when expressed as a fusion protein with GAL4 DNA-binding domain. Recently, it was reported that RAD23 interacts with yeast 26 S proteasome at least through Cim5, an ATPase subunit, via its NH_2-terminal portion (25), whereas Mcb1, an yeast counterpart of human S5a, has not been proven to interact with RAD23. It was also reported that hemagglutinin-tagged RAD23 is rapidly degraded in a 26 S proteasome-dependent manner in yeast (25). These data raise the possibility that the hHR23 proteins migrate with 26 S proteasome to be degraded as same as in yeast. However, our results propose the different possibility. We demonstrated here that hHR23B inhibited the lysozyme degradation in reticulocyte extracts without its own degradation (Fig. 3). This stability of hHR23B supports the previous finding that the native RAD23 protein is extremely stable, and Ub-like domain in RAD23 does not mediate its degradation in yeast (4). Thus we propose the model that hHR23B somehow regulates the proteolysis of the ubiquitinated protein by 26 S proteasome via the specific interaction with S5a, rather than 26 S proteasome simply degrades hHR23 proteins as its target.

The Ub-like Domain of hHR23 Proteins and the Multiubiquitin Chain-binding Domain of S5a Are Responsible for Interaction between hHR23 Proteins and S5a—We demonstrated that the Ub-like domain of hHR23B is necessary to interact directly with S5a (Fig. 4*B*, *lanes 4* and *13*). This result corresponds well with the previous finding that the Ub-like domain of hHR23B is sufficient to interact with 26 S proteasome in HeLa cell extracts (25). Our present paper is the first report to indicate clearly that one of the hHR23-interacting protein in the 26 S proteasome is S5a in mammalian cells. While rhHR23B-(1–87) protein has extra sequences (8 amino acids) beyond the Ub-like domain, we obtained evidence that the first 72 amino acids (which show 32% identity and 58% similarity with ubiquitin) are responsible for the binding with the multiubiquitin chain binding protein S5a (see Fig. 4*B*, *lane 4*). It is obvious from the previous report by van Nocker *et al*. (26) as well as our present data that S5a has only a poor affinity to the ubiquitin monomer. On the other hand, we previously reported that the majority of hHR23 proteins exist as a monomer form in cell extracts (3). Our present results indicate that the Ub-like domain of the hHR23 proteins has a high affinity for S5a as a monomer (Figs. 5 and 6*A*). In contrast to this, neither Ub-rhHR23B nor NEDD8, which shows high homology to ubiquitin, can interact with S5a (Fig. 6). These results strongly suggest that S5a has a high preference for binding to the Ub-like domain of hHR23 proteins. Watkins *et al*. (4), however,

previously reported that Ub-RAD23 can functionally replace the UV damage response of the native RAD23 protein in yeast. Therefore, it is also likely that Ub-hHR23B is able to substitute the physiological function of native hHR23B in human cell. In addition we mapped the hHR23-binding region in S5a. So far, the S5a homolog has been isolated from *S. cerevisiae*, *Arabidopsis*, *Drosophila*, mouse, and human (23, 26–30), and several lines of evidence have been obtained that S5a is a multiubiquitin chain-binding protein. Based on amino acid sequence alignments, it was noted that S5a homologs harbor a highly conserved sequence starting from the specific tetrapeptide, GVDP (23). Two repeats of this sequence were identified in higher eukaryotic proteins, while the *S. cerevisiae* S5a homolog has only one. The hHR23-binding region comprised of 81 amino acids was mapped between the GVDP tetrapeptide repeats in S5a (Fig. 7). Recently, three independent groups reported the identification of the multiubiquitin chain-binding site in S5a homologs (31–33). Especially, two multiubiquitin-binding sites were identified in human S5a, designated as PUbS1 (Met^{196}-Ala^{241}) and PUbS2 (Met^{263}-Asp^{307}) (33). PUbS1 has a GVDP tetrapeptide and a conserved hydrophobic amino acid stretch. PUbS2 lacks the GVDP tetrapeptide but has a hydrophobic amino acid stretch which is also conserved among higher eukaryotes. PUbS2 was shown to exhibit higher affinity for multiubiquitin chain compare with PUbS1 (33). Surprisingly, the region responsible for the interaction with hHR23 proteins in S5a is identical to the sequence of PUbS2 (Fig. 8*A*). These results indicate that a monomer of the Ub-like domain of hHR23B is able to bind to the multiubiquitin chain-binding region of S5a and support our hypothesis that hHR23 proteins might be competitive with multiubiquitin chain. Our experiments using a series of deletion mutants of hHR23-binding domain of S5a indicate further that the stretch of amino acids NH_2-terminal to the hydrophobic region (IAYAM) are necessary for binding activity (Fig. 8, *B* and *C*, *lanes 4* and *5*). It was reported that the presence of amino acids NH_2-terminal to the hydrophobic region increased the amount of multiubiquitin chain bound for PUbS2 (33). These results thus indicate that the site for hHR23-binding overlaps with the site for multiubiquitin-binding in S5a. However, it remains to be determined whether the amino acids NH_2-terminal to the hydrophobic region of S5a are sufficient to interact with the hHR23 proteins, and whether hHR23 proteins and the ubiquitin polymer could compete with each other for binding to S5a. These issues are now under investigation in our laboratory.

It is well known that DNA damage causes a significant problem for eukaryotic cells (34). To facilitate repair processes prior to DNA replication and cell division, the cell cycle is rigidly controlled in response to DNA damage. Proteolytic process is one pathway to restrict the cell cycle (35). It is obvious that the Ub-like domain of RAD23 is responsible both for UV damage response (4) and to interact with 26 S proteasome in yeast (33). From the strong conservation in structure and function of yeast and human repair factors, it is likely that the Ub-like sequence of hHR23 proteins is also responsible for UV damage response in mammalian cells via the proteolytic pathway.

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