hHR23B is the human homologue of the yeast protein RAD23 and is known to participate in DNA repair by stabilizing xeroderma pigmentosum group C protein. However, hHR23B and RAD23 also have many important functions related to general proteolysis. hHR23B consists of N-terminal ubiquitin-like (UbL), ubiquitin association 1 (UBA1), xeroderma pigmentosum group C binding, and UBA2 domains. The UBA domains interact with ubiquitin (Ub) and inhibit the assembly of polyubiquitin. On the other hand, the UbL domain interacts with the poly-Ub binding site 2 (PUbS2) domain of the S5a protein, which can carry polyubiquitinated substrates into the proteasome. We calculated the NMR structure of the UbL domain of hHR23B and determined binding surfaces of UbL and Ub to UBA1, UBA2, of hHR23B and PUbS2 of S5a by using chemical shift perturbation. Interestingly, the surfaces of UbL and Ub that bind to UBA1, UBA2, and PUbS2 are similar, consisting of five β-strands and their connecting loops. This is the first report that an intramolecular interaction between UbL and UBA domains is possible, and this interaction could be an important for the control of proteolysis by hHR23B. The binding specificities of UbL and Ub for PUbS1, PUbS2, and general ubiquitin-interacting motifs, which share the LALA motif, were evaluated. The UBA domains bind to the surface of Ub including Lys-48, which is required for multiubiquitin assembly, possibly explaining the observed inhibition of multiubiquitination by hHR23B. The UBA domains bind to UbL through electrostatic interactions supported by hydrophobic interactions and to Ub mainly through hydrophobic interactions supported by electrostatic interactions.

hHR23B is the human homologue of the yeast protein RAD23. hHR23B was originally characterized as a protein that complements the xeroderma pigmentosum group C protein (XPC), which recognizes DNA damage so as to initiate nucleotide excision repair (1–4). hHR23B participates directly in nucleotide excision repair by forming a complex with XPC. The loss of yeast homologue of hHR23B results in decreased nucleotide excision repair activity, and it is likely that RAD23 stabilizes Rad4 (the yeast homologue of XPC), which is otherwise continually degraded by ubiquitin-dependent proteolysis (5, 6). Experiments with knockout mice carrying a homozygous loss of the gene encoding either mHR23A or mHR23B showed that these two proteins are functionally redundant in terms of response to DNA damage by UV light. However, the double knockout mouse was not viable, and only cell lines were established from the embryos. Interestingly, the XPC protein is absent from the double knockout cells but could be detected again after treatment with a proteasome inhibitor. The mHR23B knockout mouse, but not the mHR23A knockout, showed defects in postnatal growth, suggesting that hHR23B may have functions beyond those related to XPC and DNA repair (7). Previous studies have suggested that hHR23B also functions in the 26 S proteasome-dependent protein degradation pathway (8–11).

hHR23B has four well defined functional domains, including the N-terminal ubiquitin-like (UbL), ubiquitin association 1 (UBA1), XPC binding (XPCB), and ubiquitin association 2 (UBA2) domains, which are linked by highly flexible Gly-rich loops (2, 12). Deletion of the UbL domain of RAD23 in yeast gives rise to a sensitivity to UV irradiation that was intermediate between those of wild-type and ΔRAD23 strains (5, 13). RAD3 and DD1 were originally identified as the suppressors of a temperature-sensitive pds1 mutant; the pds1 gene encodes a protein that controls the late S phase checkpoint in yeast and becomes polyubiquitinated and degraded. The Pds1 temperature-sensitive mutant protein is more rapidly degraded than the wild type in non-permissive temperature. However, the UBA domains of RAD3 and DD1 can suppress degradation of the Pds1 temperature-sensitive mutant protein by preventing polyubiquitination (9). RAD23 and DD1 share common motifs, including the N-terminal UbL and C-terminal UBA domains, and could form both homo- and heterodimers (14, 15). Another binding study using surface plasmon resonance showed that the fission yeast protein Mud1 (the orthologue of budding yeast DD1) has a higher affinity for tetra-Ub than for mono-Ub (16).

The UbL domain of hHR23B has a high binding affinity for the polyubiquitin binding site 2 (PUbS2) of the human S5a protein (12, 17). S5a has two well conserved mult ubiquitin binding sites (PubS1 and PubS2) and displays a much higher affinity for octa-Ub than for tetra-Ub (18, 19). S5a is the only

XPCB, XPC binding; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; H$^\text{31} \text{N}$ single-quantum correlation.
component of the proteasome that can reside outside of the 26 S proteasome complex, and it is supposed to be a shuttle delivering polyubiquitinated, degradable protein substrates into the proteasome (12, 18). Deletion of the yeast homologue of S5a (Rpn10) from wild-type yeast yields no serious biological defects; however, the ΔRpn10/ΔRAD23 double mutant displays growth and proteolytic defects (20, 21). Taking into account all these data, the current model of RAD23 function is that RAD23 delivers polyubiquitinated degradable proteins into the proteasome in an Rpn10-dependent manner (8, 11, 20, 22). Watkins et al. (13) showed that the UBL domain of RAD23 could be functionally replaced by Ub in yeast. Recently, it was shown that the N-terminal Ub of Ub-RAD23, in which the UBL domain is replaced by Ub, is mult ubiquitinated in vivo, and this promotes the binding of Ub-RAD23 to S5a (23).

Many studies have been performed to attempt to elucidate the function of hHR23B (RAD23) in 26 S proteasome-dependent proteolysis, but a precise pathway could not be determined until now. Here, we use NMR techniques to study the three-dimensional structure of the UBL domain of hHR23B and the individual interactions among UBL, UBA1, UBA2, S5a of PUbS2, and ubiquitin, including the determination of binding constants for each domain. We were interested in determining the mode of hHR23B-dependent regulation in protein degradation and in elucidating why hHR23B has both a UBL and UBA domain. From our studies, we observed clear domain-domain interactions between (i) UBL and UBA1 and UBA2; (ii) Ub and UBA1 and UBA2; and (iii) Ub, UBL, and PUbS2 of S5a.

EXPERIMENTAL PROCEDURES

Clonings and Protein Purification—The genes that encode hHR23B and the PUbS2 of S5a (81 amino acids, 263–343) were generously provided by Prof. Hanaoka. The UBL domain (82 amino acids, 1–82) was subcloned into the PET15B vector (NewBamHI). The UBA1 (49 amino acids, 185–233) and UBA2 (50 amino acids, 361–410) domains, as well as two versions of the PUbS2 of S5a (45 and 53 amino acids, 263–307 and 263–315), were subcloned into the pGEX-4T3 vector (BamHI/XhoI) for high levels of expression. PUbS2 of S5a (an 81-amino-acid fragment) has a trimeric hydrodynamic molecular weight from gel permeation chromatography (actually, it is dimeric), but 45-amino-acid and 53-amino-acid forms showed the expected molecular weight in gel permeation chromatography. The Ub gene was isolated from a mammalian expression vector with the hemagglutinin-tagged Ub gene, which was a gift from Dr. Daeyoup Lee, and subcloned into pET15B using the same restriction enzyme sites as for the UBL domain. The correct subclonings were confirmed by DNA sequencing.

The Ub and UBL domains were purified using a His tag column (Qiagen). UBA1, UBA2, and PUbS2 (the 45-amino-acid and 53-amino-acid fragments) were purified using a glutathione S-transferase affinity column (Amersham Biosciences), and the N-terminal His tag and glutathione S-transferase were removed also by thrombin digestion. All domains were further purified using a Superdex75 column (Amersham Biosciences). For preparation of isotope-labeled proteins, we used M9 minimal media supplemented with a vitamin mixture and trace metal solutions. In the case of PUbS2, we used the 53-amino-acid fragment for further experiments because it had higher expression level and stability during the purification.

NMR Experiments and Structure Calculation—NMR samples were prepared in the buffer solution containing 7.5% D2O (50 mM sodium phosphate and 100 mM sodium chloride, pH 6.0). In the case of UBA2, we added 2 mM dithiothreitol to prevent the intermolecular disulfide bridge. All NMR spectra were recorded using a 900-MHz Varian Unity Plus spectrometer equipped with a 7.0-Tesla spectrometer. The NOE distance restraints from the NOESY-N15-HSQC and two-dimensional D2O-N15-HSQC, and we extracted the dihedral angle restraints from TALOS chemical shift analysis (24) and intensity-modulated N15-HSQC (25). The dihedral angle (θ) was extracted from the J-coupling constant using the Karplus equation: \(J(θ) = A \cos(θ - 60) + B \cos(θ - 60) + C\), where \(A = 6.51\), \(B = -1.76\), and \(C = 1.60\) (26). Structure calculation was performed using Cytana, which combines automated assignment of NOE cross-peaks and structure calculation (27). The UBL structures from Cyana already had a good pairwise backbone root mean square deviation (0.44 Å) and good target functions, but the C-terminal flexible part of the calculated structures is very close to one long α-helix, although there were no distance restraints in that region. We performed further structure calculation (restraint molecular dynamics) using the Amber7 program in a vacuum (28) including the standard generalized Born model, which can account for the better structures. However, there were serious distance violations when we used the distance restraints from the direct Cytana output, although the distance generation program of Amber introduced the pseudotautom correction. Then we applied an ambiguity distance to the methyl protons of valine and leucine. The calculated structures were overviewed by using PROCHECK program (29).

Chemical Shift Perturbation—N15-HSQC spectra were obtained by varying the ratio of N15-labeled and non-labeled proteins. Protein concentrations were determined using a UV spectrophotometer, and the extinction coefficients of UBA1, UBA2, S5a PUbS2, and Ub were 1480, whereas that of Ub was 2960 (m−cm−1) at 280 nm (30). Although most people use the value of the square root of \(0.5 \times \Delta N\delta \Delta H\) for chemical shift presentation (17, 31), we found that the ratios between the sum of \(\Delta N\) and \(\Delta H\) of all residues varies from ~5 (N15-labeled Ub) to ~10 (N15-labeled UBL). Therefore, we added the normalized values of \(\Delta N\) and \(\Delta H\) separately for keeping the smaller signal of the \(\Delta H\) than that of \(\Delta N\), such as the percentage of \((\Delta N/2\Delta N+\Delta H)/2\Delta H\). The binding constants were extracted from the separate fitting of N15 and H chemical shifts to a simple binding equation. All binding interactions were fast exchange processes, and we could trace most of the chemical shift changes by adding counter proteins. However, some peaks with very large perturbations disappeared and reappeared during the courses of titration.

NMR Data Manipulations and Three-dimensional Visualizations—The NMR data were processed using NMRpipe (32) and analyzed using Sparky (33). All structure visualizations were performed with the Chimera (34) and MOLMOL (35) programs. Surface electric potential maps were generated with the DelPhi program using the default parameters (36).

RESULTS AND DISCUSSION

Structure of UBL Domain—We first solved the structure of the UBL domain of hHR23B so that we could more accurately analyze the interactions between the various domains that were studied here (Fig. 1). The structure of UBL is very similar to Ub and the root mean square deviation of their backbone is 1.6 Å and their secondary structures is only 0.81 Å. The overall view of structure is shown in Table 1. UBL has four large and one small β-strand (residues 1–7, residues 12–17, residues 44–48, residues 69–74, and residues 51–52, respectively), a long α-helix (residue 23–34), and one turn of α-helix (residues 59–62). There might be another short 310 helix in the vicinity of residues 41–43. The five β-strands form a platform to interact with other proteins, and the long α-helix exists on the other side of this platform. The structure of UBL shows that the UBL domain evolved from an Ub prototype, and its biological functions might be related to the original function of Ub.

Binding Surface of the UBL Domain of hHR23B—There are many reports that Ub can specifically interact with the UBA domains (15, 16, 37). Therefore, we were interested in assessing the interactions between the UBL domain, which is similar to Ub, and UBA domains of hHR23B, and also in comparisons with its binding surface for PUbS2 of S5a.

We performed chemical shift perturbation (CSP) experiments with N15-labeled UbL in the presence of UBA1, UBA2, and PUbS2 (Fig. 2). It is interesting and important to note that there is binding between UBL and UBA domains that are present in the same hHR23B protein, although their binding affinity is low (Table II). PUbS2 resulted in strong CSP of the UBL domain, in agreement with a previous report (17). We attempted to determine the binding constant for PUbS2 and UbL by titrating PUbS2 (20 μM) using CD spectroscopy, but we could not get good binding curves because this interaction is too tight. This means that its binding constant is much less than 10 μM (data not shown). It is possible to infer why UBA1 and
UBA2 have a lower binding affinity for UbL than does PUbS2 by comparing the relative heights of CSP in the specific regions (Fig. 2). There are three main chemical shift perturbed regions, at amino acid residues 1–15, 40–60, and 60–80, and all three have similar heights when the PUbS2 interacts with UbL. However, in the case of UBA1 and UBA2, the middle region has a relatively higher CSP when compared with the other two regions. Generally, the binding interface without the extraordinarily high chemical shift perturbing residues, such as a ring current generating or directly hydrogen-bonding residues, could likely produce the equal amount of CSP in the overall interface, and also the area of binding interface could be correlated to the binding strength. Moreover, Ub that has a similar binding surface and higher binding affinity for UBA domains showed the relatively equal amount of summed CSP in these three regions (Fig. 2). Therefore, this could indicate that binding of the UBA domains to UbL depends mainly on the middle region, and the CSP in the other two regions might be caused by the indirect proximity of UBA domains or by the weak interaction during the binding through the prime middle region. However, these different amounts of CSP in these three regions might result from the locally higher effects on chemical shift by UBA molecules because the binding mode of UBA domains for UbL is somewhat different from that of UBA domains to Ub (see below). The binding surface of UbL is mainly formed by the five β-strands, including the loop regions between β1 and β2 and between β3 and β4. It is obvious that residues 48–52 in the β3-bend-β4 region form a major binding surface for the UBA domains, but the binding surface of PUbS2 is well spread over all five β-strands (Fig. 3).

**Binding Surface Mapping between hHR23B, Ub, and S5a**

UBA2 have a lower binding affinity for UbL than does PUbS2 by comparing the relative heights of CSP in the specific regions (Fig. 2). There are three main chemical shift perturbed regions, at amino acid residues 1–15, 40–60, and 60–80, and all three have similar heights when the PUbS2 interacts with UbL. However, in the case of UBA1 and UBA2, the middle region has a relatively higher CSP when compared with the other two regions. Generally, the binding interface without the extraordinarily high chemical shift perturbing residues, such as a ring current generating or directly hydrogen-bonding residues, could likely produce the equal amount of CSP in the overall interface, and also the area of binding interface could be correlated to the binding strength. Moreover, Ub that has a similar binding surface and higher binding affinity for UBA domains showed the relatively equal amount of summed CSP in these three regions (Fig. 2). Therefore, this could indicate that binding of the UBA domains to UbL depends mainly on the middle region, and the CSP in the other two regions might be caused by the indirect proximity of UBA domains or by the weak interaction during the binding through the prime middle region. However, these different amounts of CSP in these three regions might result from the locally higher effects on chemical shift by UBA molecules because the binding mode of UBA domains for UbL is somewhat different from that of UBA domains to Ub (see below). The binding surface of UbL is mainly formed by the five β-strands, including the loop regions between β1 and β2 and between β3 and β4. It is obvious that residues 48–52 in the β3-bend-β4 region form a major binding surface for the UBA domains, but the binding surface of PUbS2 is well spread over all five β-strands (Fig. 3).

**Binding Surface of Ub**—Many studies showed that UBA interacts physically with Ub and the UBA domains and plays


| Table II | Binding constants |
|-----------------|------------------|
|                | $K_d$ (mM)        |
| UBA1           | 0.31 ± 0.020$^a$ |
| UBA2           | 0.36 ± 0.078$^a$ |
| PUBS2          | 0.073 ± 0.008$^a$|

$^a$ Errors show the value of standard deviation.

$^b$ Roughly estimated from the titration of PUBS2 by Ubl using CD.

an important role in the inhibition of poly-Ub chain assembly (15, 18, 37). Wilkinson et al. (16) reported that mono-Ub binds to RAD23 and DDII with a 10 $\mu$M $K_d$ through the interaction with UBA domains of these proteins, but the fission yeast MUD1, the orthologue of DDII, has a higher specificity for tetra-Ub. Therefore, we studied the binding surfaces of Ub to ascertain how the UBA domains of hHR23B inhibit multi-Ub formation and to compare it with the binding of PUBS2. In doing so, we hoped to determine the binding specificity of the hHR23B UBA domains to multi-Ub.

The CSP patterns showed that Ub binds to the UBA1 and UBA2 domains of hHR23B and to PUBS2 of S5a via the similar surface of Ubl (Fig. 2). Mappings of each chemical shift perturbed region on the NMR structure of Ub (38) show almost the same patterns as those observed in the Ubl (Fig. 3). However, Ub has no locally defined, major binding surface for UBA1 and UBA2, different from the Ubl and the larger surface of Ub, takes part in the interaction with these UBA domains. This finding is reasonable because the binding affinity for the UBA1/2-Ub interactions are high when compared with those of the Ubl-UBA interaction (Table II). The binding surface for UBA1/2 onUb is mainly formed by the same three regions as in Ubl. However, Ub showed one additional chemical shift perturbed region (residues 22–36). This region corresponded to the long $\alpha$-helix that supports the backside of five $\beta$-strands, and the CSP in the $\alpha$-helix might be the result of an indirect effect of PUBS2 binding.

We speculated that there might be a secondary binding site for UBA domain in Ub, which would explain the higher binding affinity of UBA for multi-Ub versus mono-Ub. However, we saw no differences between two distinct CSP plots of Ub in the presence of two concentrations of the UBA domains (at 1/4 CSP saturation and at close to the saturation concentration of UBA domains). Therefore, we tried to make a map of the UBA binding surface of Ub on two reported x-ray crystal structures of tetra-Ub (residues 39, 40), but we were unable to infer the reason for the higher binding affinity of the single UBA domain to tetra-Ub. Although we could not see any secondary binding site of Ub to UBA domains, it is possible that tetra-Ub forms a new binding interface with a strong binding affinity for the UBA domain by the structural rearrangement of tetra-Ub, Raasi and Pickart (10) recently reported that RAD23 specifically binds to tetra-Ub with Lys-29–Gly-76 and Lys-63–Gly-76 linkages. They also showed that UBA1 or UBA2 domain alone has a lower binding affinity for this particular tetra-Ub than the truncated form of RAD23 with both UBA1 and UBA2. By comparing these recent reports with the previous result of the fission yeast Mud1 and with our CSP data of Ub obtained with hHR23B UBA domains, we tentatively concluded as follows: the UBA domain of Mud1 might have a high binding affinity for tetra-Ub through the different binding site. However, UBA1 and UBA2 of RAD23 and hHR23B bind to the same surface of Ub that was determined here, and these two UBA domains cooperatively bind to tetra-Ub with a specific configuration (Lys-48–Gly-76 linkage).

There are many lysine residues in Ub, such as residues 6, 11, 27, 29, 33, and 63. Among these lysine residues, Lys-29 and Lys-48 produce proteolytic multiquabination, and Lys-63 produces the non-proteolytic variety (41, 42). It was reported that a point mutation at the Lys-48 position would not abolish the whole polyubiquitination process (37). However, Lys-48 is the prime residue for poly-Ub chain assembly through the Lys-48–Gly-76 isopeptide bond. Lys-48 is located in the binding surface for UBA domains and PUBS2 (Fig. 3), whereas the other key residues of Lys-29 and Lys-63, as well as other lysines, are present on the different surfaces of Ub. We showed that the UBA domains of hHR23B interact with a surface that includes Lys-48 and can block multi-Ub assembly. Also, our result could explain the exact positioning of Ub-Lys-48 for the ubiquitination by the UBA domain that is present in many ubiquitin carrier protein (E2) and ubiquitin-protein isopeptide ligase (E3) ubiquitination proteins (43).

PUBS2 Binding Surfaces of UbL and Ub—Walters et al. (17) determined the binding surface of PPLIC-2 UbL domain for S5a protein and also suggested a binding model of PUBS2 in which hydrophobic residues Tyr-289, Ala-288, Met-291, and negatively charged Glu-284, Glu-285 form a binding surface for UbL. Here, we focused on the separated PUBS2 domain instead of whole S5a protein so as to characterize the binding surfaces of hHR23B UbL and Ub in more detail. Using CD and NMR, we confirmed that PUBS2 binds to Ubl as a stable $\alpha$-helical conformation. From the backbone chemical shift assignment of PUBS2 bound to Ubl and from Talos chemical shift analysis, we defined the $\alpha$-helical region (from Glu-283 to Glu-296) that contains the Ub binding motif, IAYAM (see supplement 1). The structure calculation of this complex allowed us to go further in our conclusions. From the CSP results, we found that the main binding surfaces on PUBS2 of S5a are formed by Lys-51, Ala-49, Ile-47, Ile-52, Val-71, Leu-46, Met-73, and Leu-8 for UbL and by Lys-48, Ala-46, Gly-47, Ile-44, Gln-49, Val-70, Leu-8, and Leu-71 for Ub (Fig. 2). PUBS2 has a flat-shaped $\alpha$-helix in which a negatively charged Glu-284, Glu-285 and the hydrophobic Tyr-289, Ala-288, Met-291, and negatively charged Glu-284, Glu-285 of PUBS2 probably locate on the positively charged patches of UbL (Lys-51) and Ub (Lys-48). We found that Leu-295 is a key residue to explain a higher binding affinity of PUBS2 for UbL of hHR23B than other general Ub-interacting motifs (UIMs), such as Hrs-UIM (31). The UIM sequence shares the common motif (LALA) with PUBS1 and PUBS2. The Leu-295 is exactly $\mu$M $K_d$ than does PUBS2 (73 $\mu$M of $K_d$), has a glutamine instead of Leu-295 of PUBS2. It is interesting to note that Hrs-UIM showed an additional strong CSP in the long $\alpha$-helix of Ub (31). This is different from our cases, in which UBA1 and UBA2 showed a negligible CSP, and PUBS2 caused a much lower CSP in this $\alpha$-helix region than does Hrs-UIM. The energetically unfavorable structure change of Ub caused by the binding of the Hrs-UIM could also explain why PUBS2 of S5a has a stronger interaction with Ub than does Hrs-UIM.

From our results, we can explain why PUBS2 has a lower binding affinity for Ub (Table II) than does PUBS2. It is because Ub has a charged His-68 and a hydrophilic Gln-49 in the PUBS2 binding surface but not in the Ubl surface. Similarly, PUBS1 possibly has a lower binding affinity for UbL and Ub than does PUBS2 because PUBS1 has an arginine at position 292 instead of a glutamine that is located on the same
binding surface for UbL and Ub. The slight positive surface potential of UbL and Ub less favorably accommodates positively charged arginine.

**Binding Surfaces of UBA1 and UBA2**—It would be interesting to see which parts of hHR23B UBA1 and UBA2 interact with UbL of hHR23B and Ub, comparing them with PUBS2 of S5a because their structures are different: PUBS2 has an α-helical conformation as a binding structure; however, UBA domains have three helices, in which loop 1 between helices 1 and 2, as well as helix 3, form a large hydrophobic patch (44, 45). Therefore, we characterized the binding of the UBA domains during interaction with UbL and Ub (Fig. 2). Both UBA domains showed CSP in most of their residues in the presence of the UbL domains and Ub. The small structural changes of UBA domains are possible during the binding interaction because these are very small domains consisting of only three helices, and their rigidity might be low. For example, Ala-386 and Ala-397 of UBA2 are completely shielded by other residues, but these residues show high CSP. We represented the major binding surface of UBA1 and UBA2 using homologous modeled structures from SWISS-MODEL, an automated comparative protein modeling server (Fig. 3). These model structures are almost the same as the published NMR structures of hHR23A UBA1 and UBA2 (44, 45).

It is interesting that the characteristics of the main UbL and Ub binding surfaces of UBA domains are different. UBA domains bind with Ub mainly through hydrophobic interactions supported by electrostatic interactions (UBA1: Ile-197, Met-200, Tyr-202, Arg-220, Leu-226, and Glu-223; UBA2: Leu-373, Ala-375, Leu-376, Phe-378, Leu-396, Leu-402, and Glu-394), and the binding surfaces for Ub are similar to each other. However, their binding surfaces for the UbL domain are different, and electrostatic interaction supported by hydrophobic interactions may be important in these interactions (UBA1: Glu-223, Arg-220, Leu-226, Met-227, and Ile-229; UBA2: Gln-403, Gln-404, Asn-405, Phe-406, and Asp-407). Arg-221 of UBA1 can also contribute to hydrophobic interactions because of its long acyl chain. Electrostatic potential maps can support the electrostatic contribution to the binding of UBA domains to UbL because the surface potential of UBA domains is mostly negative, and that of UbL is positive. UBA showed 10-fold different binding affinities for UbL (−2 mM $K_d$) and Ub (−0.3 mM $K_d$), although the hydrophobic surfaces of UbL and Ub are very similar (Table II). It is probable that the binding interface between UBA domain and Ub is matched better than that of UBA and Ub. These different binding interfaces could be the cause of the weak interaction between the UbL and UBA domains. UBA2 had a relatively larger and stronger CSP than does UBA1 when bound to UbL, and this could be the source of the slight increased binding affinity of UBA2 for UbL. It is interesting that N-terminal residues of UBA1 (Leu-184, Val-185, and Thr-186) and C-terminal residues of UBA2 (Gln-403, Gln-404, Asn-405, Phe-406, and Asp-407) showed an appreciable CSP, although this region of solution structures does not
have a defined structure (44, 45). However, these regions are well conserved among the RAD23 homologues, and these suggested binding surfaces are reasonable.

Biological Implications of UbL-UBA Intramolecular Interaction—There have been no reports about intramolecular interactions between UbL and the UBA domains of RAD23 homologues since this protein was shown to be involved in proteasome-dependent protein degradation. As mentioned above, all domains of hHR23B are linked by unstructured residues, especially the Gly-rich loop between XPCB and UBA2. We observed a well defined interaction between separated UbL and UBA domains from hHR23B.

If this interaction were an intramolecular process, it would be hard to imagine its biological function because it is too weak. However, the UbL and UBA domains are subdomains of one protein, hHR23B, and an intramolecular interaction is not concentration-dependent. When an intramolecular interaction is strong, it is almost impossible to break. Therefore, the weak intramolecular interaction that we observed becomes more significant in a real biological system. Also, the observation that the chemical shift perturbed regions of hHR23B UbL by the UBA domains is similar to those by the PUBs2 of S5a supports the hypothesis that RAD23 promotes the targeting of UbL-bound proteins to the proteasome pathway, but DDI1 has a less conserved UbL domain.

It has been suggested that RAD23B promotes the targeting of proteolytic substrates to the proteasome, and high expression of the UbL domain retards proteolysis of the model substrate Ub-Pro-β-galactosidase (8). The exposed UbL domain of hHR23B can interact with PUBs2, and this non-functional interaction might result in unfavorable consequences to other basal level proteolysis. The intradomain sequestering of UbL interaction might result in unfavorable consequences to other, but that structurally distinct UBA and PUBs2 domains bind to the same surfaces of UbL domain and Ub. During intracellular processes, differently structured domains, such as UBA and PUBs2, could converge upon the same molecules to interact with the common binding surface therein; thus, competition may occur between these two domains. We also observed that slight changes in amino acid sequences within their homologous sequences regulate the binding affinities of the various UIM, PUBs1, PUBs2, and UBA domains.

Finally, we put forth our fluid hypothesis. UbL of hHR23B is shielded by the UBA domains to reduce the unfavorable interactions with S5a. However, under the appropriate circumstances, such as the accumulation of multi-Ub or XPC protein, the UbL domain can open by the UBA-multi-Ub binding or the XPC-XPCB domain binding. At this time, we prevent the poly-Ub chain assembly, and UbL interacts with S5a to inhibit the innate S5a function of basal level proteolysis. However, the small moiety of Ub (tetra-Ub) can not replace the S5a-UbL interaction because of its lower binding affinity for the whole S5a protein. When the higher molecular weight poly-Ub accumulates, these poly-Ub moieties can replace the UbL bound to S5a because octa-Ub has a higher affinity for S5a than does tetra-Ub (47). This process might be accompanied by the structural change in S5a. When poly-Ub accumulates, hHR23B could then serve as a shuttle for polyubiquitinated proteolytic substrates in an S5a-dependent manner.

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FIG. 4. Summary of All Interactions

We first showed that UbL and UBA domains in the same protein (hHR23B) interact with each other, but that structurally distinct UBA and PUBs2 domains bind to the same surfaces of UbL domain and Ub. During intracellular processes, differently structured domains, such as UBA and PUBs2, could converge upon the same molecules to interact with the common binding surface therein; thus, competition may occur between these two domains. We also observed that slight changes in amino acid sequences within their homologous sequences regulate the binding affinities of the various UIM, PUBs1, PUBs2, and UBA domains.

Finally, we put forth our fluid hypothesis. UbL of hHR23B is shielded by the UBA domains to reduce the unfavorable interactions with S5a. However, under the appropriate circumstances, such as the accumulation of multi-Ub or XPC protein, the UbL domain can open by the UBA-multi-Ub binding or the XPC-XPCB domain binding. At this time, we prevent the poly-Ub chain assembly, and UbL interacts with S5a to inhibit the innate S5a function of basal level proteolysis. However, the small moiety of Ub (tetra-Ub) can not replace the S5a-UbL interaction because of its lower binding affinity for the whole S5a protein. When the higher molecular weight poly-Ub accumulates, these poly-Ub moieties can replace the UbL bound to S5a because octa-Ub has a higher affinity for S5a than does tetra-Ub (47). This process might be accompanied by the structural change in S5a. When poly-Ub accumulates, hHR23B could then serve as a shuttle for polyubiquitinated proteolytic substrates in an S5a-dependent manner.
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