Article

Rwdd1, a Thymus Aging Related Molecule, Is a New Member of the Intrinsically Unstructured Protein Family

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We had previously identified a novel protein termed Rwdd1 whose expression in thymus is decreased in aged or oxidatively stressed mice. In the present study, we found that Rwdd1 expressed in both prokaryotic and eukaryotic cells showed a slower migration rate on SDS-PAGE gel. In addition, Rwdd1 was more sensitive to proteinase proteolysis. Furthermore, being a highly acidic protein which contains an RWD domain, Rwdd1 shared a high level of sequence similarity with Gir2, a member of the intrinsically unstructured protein (IUP). These findings suggest that Rwdd1 is a novel member of the IUP family. *Cellular & Molecular Immunology*. 2008;5(5):333-339.

Key Words: Rwdd1, intrinsically unstructured protein, Gir2

Introduction

Rwdd1 is a thymus aging-related protein which contains an RWD domain at its N-terminus. In our previous study, we found that Rwdd1 was expressed in both thymocytes and thymic epithelial cells and located primarily in the cytoplasm. Rwdd1 expression in thymus was decreased in aged and oxidatively stressed mice (1).

In order to further understand the function of Rwdd1, His-tagged Rwdd1 fusion protein were expressed in *Escherichia coli*. Surprisingly, the recombinant protein showed an abnormally slow migration behavior in SDS-PAGE. This phenomenon was also seen in the *Escherichia coli* expressed GST-tagged as well as the eukaryotic cell expressed Rwdd1.

To explore the basis of this phenomenon, we compared the molecular characteristics of Rwdd1 with three previously reported RWD containing proteins, including GCN2 (Gen Bank accession No. XP_192908), Gir2 (GenBank accession No. NP_010436.1) and RSUME (GenBank accession No. CAG38524). It was found that Rwdd1 had a similar amino acid composition as Gir2, a member of intrinsically unstructured protein (IUP) family which also showed abnormal electrophoresis behavior (2). Therefore, biophysical characteristics of Rwdd1 were further illuminated by using approaches such as folding status prediction, mass spectrometry, and proteinase proteolysis. Our results suggest that Rwdd1 has the characteristic of a natively unfolded structure.

Materials and Methods

Plasmid constructions

For prokaryotic expression, the full-length Rwdd1 coding sequence was PCR-amplified using thymic cDNA as template and cloned upstream of the His tag coding region in pET30 vector or downstream of the glutathione-S-transferase (GST) coding region in pGEX-4T-1 vector (Amersham). The N-terminal 148aa coding region and C-terminal 114aa coding region were subcloned in pET30 or pGEX-4T-1. For eukaryotic expression, the full-length or truncated Rwdd1 coding sequence were cloned in pcDNA3.1/V5-HisA(+) (Invitrogen) or pEGFP-N1 (Clontech). All the inserts were thoroughly sequenced. The sequences of designed PCR primers were listed in Table 1.

Prokaryotic expression of full-length and N- or C-terminal portion of Rwdd1

Prokaryotic expression plasmids encoding the full-length and truncated Rwdd1 were used for transformation of competent *Escherichia coli* BL21/DE3 cells. Transformants were cultured at 37°C overnight, diluted 1/100 (v/v), grown to OD_{600} 0.6, and then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside and harvested after 4 hours. The cell pellets were sonicated on ice in phosphate buffer solution

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Table 1. PCR primers used for the construction of Rwdd1 prokaryotic and eukaryotic expression vectors

Recombinant plasmids	PCR Primers	Sequences
Rwdd1-His6	Rwdd1-His6-fw	5'-GGAATTCCATATGACAGATTACGGCGAGGAAC-3'
	Rwdd1-His6-rv	5'-CCGCTCGAGGTCTGAGGAGTCACTCCCTGG-3'
$Rwdd1_{1-148}$-His6	$Rwdd1_{1-148}$-His6-fw	5'-GGAATTCCATATGACAGATTACGGCGAGGAAC-3'
	$Rwdd1_{1-148}$-His6-rv	5'-CCGCTCGAGAATCGTAACAGGGGTGCCATG-3'
$Rwdd1_{130-243}$-His6	$Rwdd1_{130-243}$-His6-fw	5'-GGAATTCCATATG AAGGAAGCAGAAGAAGCAGAGAAG-3'
	$Rwdd1_{130-243}$-His6-rv	5'-CCGGAATTCGTCTGAGGAGTCACTCCCTGG-3'
pGEX-Rwdd1	GST-Rwdd1-fw	5'-CGCGGATCCATGACAGATTACGGCGAGGAAC-3'
	GST-Rwdd1-rv	5'-CCGCTCGAGTCAGTCTGAGGAGTCACTCCCT-3'
pGEX-$Rwdd1_{1-148}$	GST-$Rwdd1_{1-148}$- fw	5'-CGCGGATCCATGACAGATTACGGCGAGGAAC-3'
	GST-$Rwdd1_{1-148}$- rv	5'-CCGCTCGAGAATCGTAACAGGGGTGCCAT-3'
pGEX-$Rwdd1_{130-243}$	GST-$Rwdd1_{130-243}$-fw	5'-CGCGGATCCAGGAAGCAGAAGAAGCAGAGAAG-3'
	GST- $Rwdd1_{130-243}$- rv	5'-CCGCTCGAGTCAGTCTGAGGAGTCACTCCCT-3'
pcDNA3.1-Rwdd1-V5	Rwdd1-V5-fw	5'-CGCGGATCCACGATGACAGATTACGGCGAG-3'
	Rwdd1-V5-rv	5'-CCGGAATTCGTCTGAGGAGTCACTCCCTGG-3'
pcDNA3.1-$Rwdd1_{1-148}$-V5	$Rwdd1_{1-148}$-V5-fw	5'-CGGGATCCGCCACGATGACAGATTACGGC-3'
	$Rwdd1_{1-148}$-V5-rv	5'-CCGCTCGAGAATCGTAACAGGGGTGCCATG-3'
pcDNA3.1-$Rwdd1_{130-243}$-V5	$Rwdd1_{130-243}$-V5-fw	5'-CGGGATCCCCACGATGGAAAAGGAAGCAGAAGAAGCAG-3'
	$Rwdd1_{130-243}$-V5-rv	5'-CCGCTCGAGGTCTGAGGAGTCACTCCCTGG-3'
pEGFP-N1-Rwdd1	Rwdd1-GFP-fw	5'-CCGGAATTCCGATGACAGATTACGGCGAG-3'
	Rwdd1-GFP-rv	5'-CGCGGATCCTCTGAGGAGTCACTCCCTGG-3'

supplemented with protease inhibitors cocktail (Sigma) and centrifuged. The soluble fraction was used for SDS-PAGE and analyzed by Commassie Blue staining.

Eukaryotic expression of full-length and N- or C-terminal portion of Rwdd1 and Western blot

Human embryonic kidney cell line 293T (ATCC CRL-11268™**)** was maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco). Cells were transfected with pcDNA3.1-Rwdd1-V5, pcDNA3.1-$Rwdd1_{1-148}$-V5 or pcDNA3.1-$Rwdd1_{130-243}$-V5 using lipofectamine2000™ (Invitrogen). Forty-eight hours later, transfected cells were lysed on ice in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) with protease inhibitor cocktail and centrifuged. The soluble fraction was then separated by SDS-PAGE, followed by transferring to nitrocellulose Hybond™ membrane (Amersham) using a semi-dry transfer system. Membranes were blocked with 5% nonfat milk in TBST buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.05% Tween) at room temperature for 1 h. Then the membrane was immunoblotted with anti-V5 primary antibody (Invitrogen) for 2 h at room temperature, followed by incubation with peroxidase-conjugated secondary antibody (Vector) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) reagents (PIERCE).

Purification, thrombin cleavage of GST-Rwdd1 fusion protein and molecualr mass determination by mass spectrometry

GST fusion proteins were purified and cleaved by thrombin according to the manufacturer's protocol. Briefly, Glutathione sepharose beads (GE Healthcare) were incubated with the recombinant cell lysates at 4°C for 2 h and washed thoroughly with washing solution of decreasing salt concentration (50 mM Tris HCl, pH 7.5, 1 mM DTT, 1 M-100 mM NaCl). GST fusion proteins were cleaved on beads by thrombin at 22°C for 2 h. Then the supernatants were applied to a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Autoflex MALDI-TOF-MS, Bruker) to determine the molecular mass of the target proteins.

Limited digestion by proteinase K

Beads bound GST or GST-Rwdd1 were eluted by reduced glutathione, dialyzed in 20 mM Tris-HCl (pH 8.0) and then subjected to proteinase K degradation as described (3). The ratios of GST or GST fusion protein to proteinase K were in the range of 1:100 to 1:6,000. The proteolysis reaction proceeded at 22°C for 20 min, 40 min or 60 min. And then the reaction was stopped by adding SDS-PAGE sample buffer and boiling. The protein samples were loaded for SDS-PAGE and the following Western blot using anti-GST (Santa Cruz) as the primary antibody.

Bioinformatic analysis of Rwdd1

The folding status of Rwdd1 was predicted by using FoldIndex (http://bioportal.weizmann.ac.il/fldbin/findex) (4). The sequence similarity between Rwdd1 and Gir2 was compared by using DNAMAN 5.2.10 (Lynnon Biosoft).

Results

Electrophoresis migration behavior of Rwdd1

The predicted molecular mass of Rwdd1 is 27 kDa, however,

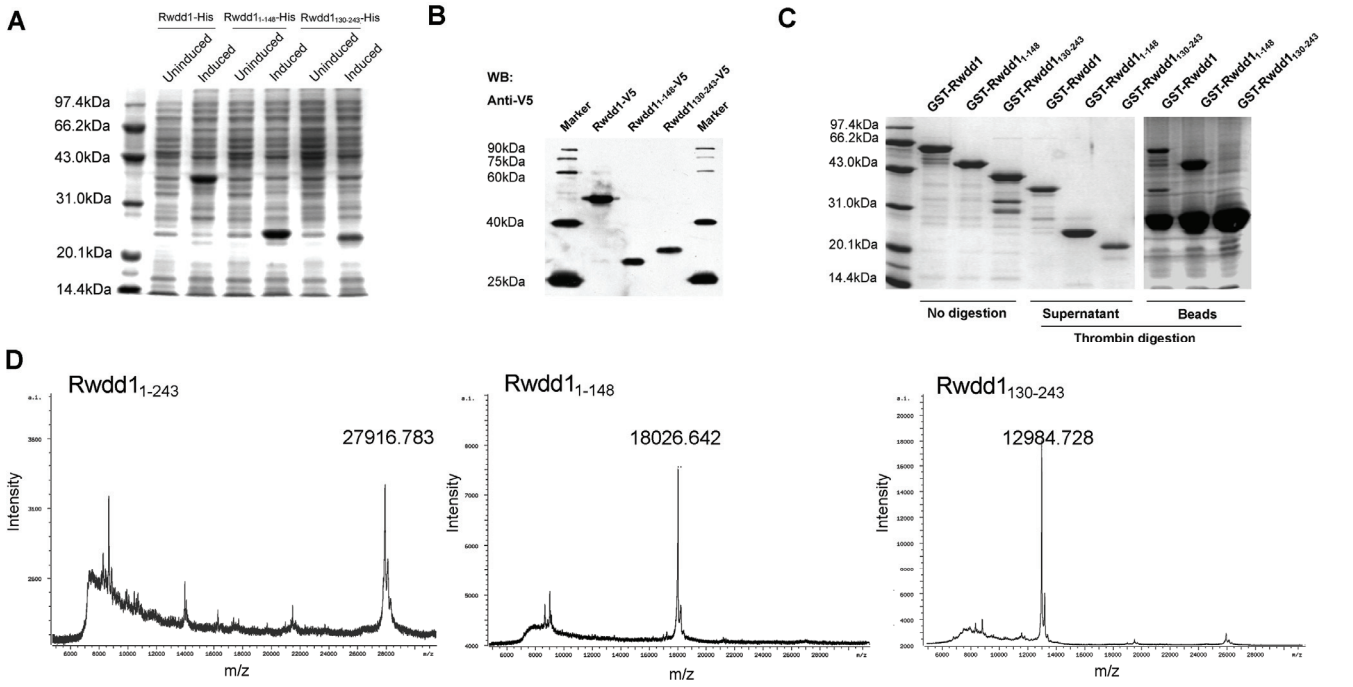


Figure 1. Prokaryotic and eukaryotic expression of full-length or truncated Rwdd1. (A) DNA fragment encoding the Rwdd1 full-length, N-terminal (Rwdd1$_{1-148}$) or C-terminal (Rwdd1$_{130-243}$) portion was cloned into pET30 vector. The recombinant plasmid was used to transform *E.coli* BL21/DE3. The expression of His tagged Rwdd1 or truncated Rwdd1 was induced by IPTG. (B) Rwdd1 or its truncated forms was cloned into eukaryotic expression vector pcDNA3.1/V5-His. Their relative expressions in transfected 293T lysates were detected by Western Blot. (C) GST-Rwdd1 or GST and truncated Rwdd1 fusion proteins were purified by glutathione sepharose beads and subjected to thrombin cleavage. (D) The molecular mass of Rwdd1, Rwdd1$_{1-148}$ and Rwdd1$_{130-243}$ that cleaved and released from their respective GST fusion proteins was analyzed by mass spectrometry.

the prokaryote expressed Rwdd1-His protein migrated as a 37 kDa protein, larger than the expected value. To further characterize Rwdd1, we constructed a series of prokaryotic and eukaryotic expression plasmids that express the full-length, N- (Rwdd1$_{1-148}$) or C- (Rwdd1$_{130-243}$) terminal portion of Rwdd1 (Figures 1A-1C). We compared the predicted size of Rwdd1 to its actual molecular mass observed in SDS-PAGE after expressed in prokaryotic or eukaryotic plasmids (Table 2). Both N- and C-terminal portions of Rwdd1 exhibited different sizes from its predicted forms. It should be noted that the tag of the fusion protein may influence the electrophoresis behavior of the target protein. Rwdd1, when expressed as a fusion protein with highly folded GST, had an electrophoresis size closer to the predicted one. However, V5-tagged Rwdd1 or truncated Rwdd1 expressed in 293T cells had larger molecular mass than expressed in bacteria, suggesting that Rwdd1 may be subjected to protein modification in eukaryotes.

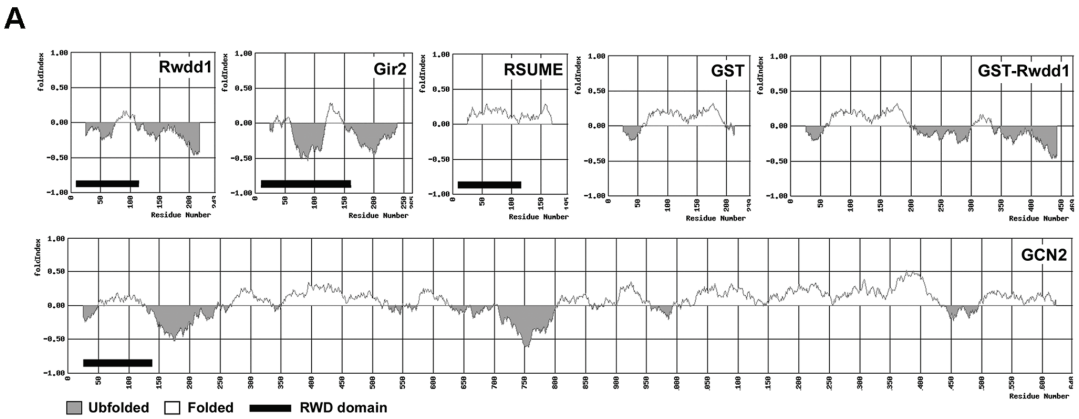
To exclude the possible interference from tag, the Rwdd1-GST fusion proteins were cleaved by thrombin to remove the GST-tag. The released proteins were subjected to SDS-PAGE and mass spectrometry to evaluate their molecular mass (Figures 1C, 1D). Full-length and truncated Rwdd1 detected by mass spectrometer had molecular mass consistent with the predicted sizes although they exhibited changed migration behavior in SDS-PAGE.

Disorder prediction of Rwdd1 and three RWD containing proteins

To investigate the sequence characteristics of Rwdd1, we compared four RWD containing proteins. Among them,

Table 2. Expected and SDS-PAGE detected molecular mass of the N- and C-terminal portions and the full-length Rwdd1 protein

Full-length or portions of Rwdd1	Molecular mass (kDa)	
	Expected	SDS-PAGE
Rwdd1	27.4	36.0
Rwdd1$_{1-148}$	16.9	23.5
Rwdd1$_{130-243}$	12.9	20.6
Rwdd1-His6	28.2	37.2
Rwdd1$_{1-148}$-His6	17.7	24.4
Rwdd1$_{130-243}$-His6	13.7	23.0
GST-Rwdd1	53.7	58.0
GST-Rwdd1$_{1-148}$	43.2	48.2
GST-Rwdd1$_{130-243}$	39.2	39.6
Rwdd1-V5	30.7	48.5
Rwdd1$_{1-148}$-V5	20.2	28.8
Rwdd1$_{130-243}$-V5	16.2	31.6



B

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Rwdd1     MTDYGEEQRNELEALESIYPDSFTVLSESPP..SFTITVTSEAGENDETVQTT......L      1
            || |||:.|||.|||||||.: ::.:. |  .|.:.:. | :..|.|   |
Gir2      .MDYKEEQKQELEVLESIYPDELRIINDEYPKIKFEVAIKLELDTGDSTSVLTKEHTIIA      1

Rwdd1     KFTYSEKYPDETPLYEIFSQE.NLEDNDVSDILKLLALQAEEN....LGMVMIFTLVTAV     53
          .|...|.||||..|..: .|| .|:||: .:  .  .:: :::    | ..  :. :...
Gir2      EFKLPENYPDEPCLISLEAQEVALNDNEEDNEEDEDEVEYDDHGNKVLKKFENLPDLISF     60

Rwdd1     QEKLNEIVDQIKTRREEEKKQKEKEAEEAEKKLFHGTPVTIENFLS.WKAKFDAELLEIK    108
          .: | |:. |:... |.:.    . . .  ..: . ..  ..: |. :. .:: |  | .
Gir2      KGYLPELTVQLESQIETDMLLGMQMCFALISSIKERCEQWYSEQLNKLEKQYELEAQERE    120

Rwdd1     KK...RMKEEEQAGKNKLSGKQLFETDHNLDTSDIQFLEDAGNNVEVDESLFQE..MDDL    167
          ||   :..:.. . .. |. :  |  : .||..|     .| :.  .:. :|::  ::.
Gir2      KKEQAKFHGTKVTRESYLEWRSKFRQELKLDERDQVRRMKAHHGKLTGKQMFEQGVVGTG    180

Rwdd1     ELEDGEDDPDYNPVAPGSDSSD....                                      222
          :   :|||:. :.||.| ...:
Gir2      DEYMEEDDASVDDVAKGLAKTEIANQ                                      240

                  I Identical residues        : and · Similar residues
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Figure 2. Comparison of different RWD containing proteins. (A) The amino acid sequences of four RWD containing proteins (including Rwdd1, Gir2, RSUME and GCN2) were submitted to http://bioportal.weizmann.ac.il/fldbin/findex. The predicted unfolded and folded regions were shown. The RWD domain was also indicated. (B) Rwdd1 (*M. musculus*, GenBank accession No. NP_079890.1) and Gir2 (*Saccharomyces cerevisiae,* GenBank accession No. NP_010436.1) were subjected to multiple protein sequence alignment. Identical and similar residues were shown separately.

GCN2 and RSUME (Rwdd3) have a nearly neutral pI value and normal electrophoresis migration behavior, whereas Rwdd1 and Gir2 are two highly acidic proteins with increased molecular mass observed by SDS-PAGE (Table 3). Rwdd1 and Gir2 had similar folding status as predicted by FoldIndex. It was shown that except for one folded region existing in the C-terminus of RWD domain, Rwdd1 was almost entirely in an unfolded state (Figure 2A). On the contrary, this configuration was not observed in GCN2 and RSUME. In addition, Rwdd1 and Gir2 also had similar sequence: identical residues = 23.14% (56/242), similar residues = 49.17% (119/242) (Figure 2B).

Limited proteolysis of GST-Rwdd1 fusion protein

GST and GST-Rwdd1 fusion proteins were subjected to limited proteinase digestion in order to further evaluate their folding status. Compared to GST, GST-Rwdd1 was extremely sensitive to proteolysis (Figures 3A, 3B). In addition, the GST part of GST-Rwdd1 fusion protein was much more resistant to proteolysis as detected by Western blot using anti-GST as the primary antibody (Figure 3C).

Discussion

IUP is a group of proteins that exist in the disordered state. Accumulating evidence suggests that the proteins could perform their functions even through in the disordered state (8). Bioinformatic analysis suggests that a substantial number of proteins might belong to IUP. Furthermore, there is increased proportion of disordered or partially disordered proteins in the eukaryotes as compared to the prokaryotes,

Table 3. Molelcular characteristics of four RWD containing proteins

Protein	Origin	Length (amino acid residue)	Content of acidic amino acids (%)	pI	Net charge (pH7.0)	Molecular mass (kDa)		Three dimensional structure	Function
						Expected	SDS-PAGE		
GCN2 (XP_192908)	*Mus musculus*	1648	15.0	6.30	-21.20	186.5	180 (5)	RWD domain: α + β sandwich fold (αββββαα) (6)	α-subunit of the translation initiation factor (eIF2α) kinase (6)
Gir2 (NP_010436.1)	*Saccharomyces cerevisiae*	265	24.5	4.22	-29.85	31.0	40-60 kDa smear (2)	Intrinsically unstructured protein (IUP) (3)	NA
RSUME (RWDD3) (CAG38524)	*Homo sapiens*	195	13.9	6.15	-3.44	22.1	21 (7)	NA	Interacts with Ubc9, involved in sumoylation (7)
Rwdd1 (NP_079890.1)	*Mus musculus*	243	25.5	3.90	-37.48	27.4	36	Supposed to be IUP	Might be involved in thymus involution (1)

which might be due to the greater need for protein-mediated signaling, regulation and control in the former (9). The major function of IUP is molecular recognition (10).

Rwdd1 is a novel RWD domain containing protein with anomalous electrophoresis behavior. RWD domain refers to three signature motifs in proteins: RING finger, WD-repeats, and yeast DEAD (DEXD)-like motif. The function of this domain is not clear enough. Present evidence suggests that RWD domain might be necessary for protein-protein interaction (11). By comparing Rwdd1 with three RWD domain containing proteins, we found that Rwdd1 was similar to Gir2 in both amino acid composition and electrophoresis behavior. Gir2, a member of IUP family, is also a highly acidic protein which is known for its unfolded structure (2, 3). The disorder predication results showed the same pattern of disorder between Rwdd1 and Gir2. In contrast, the full-length of RSUME is predicted to be folded. Similarly, the unfolded regions in GCN2 account for only a small portion of the whole protein, and locate outside the functional domains. Furthermore, the folded structure of the RWD domain of GCN2 has been confirmed by NMR (6). Both RSUME and GCN2 show normal electrophoresis behavior.

As a consequence, our question is whether Rwdd1 belongs to IUP, and whether its uncommon electrophoresis migration results from lacking a stable three-dimensional structure. Our data showed that Rwdd1 had the following characteristics of IUP: abnormal electrophoresis migration behavior; sensitive to proteinase digestion; normal molecular mass detected by mass spectrometry (3). Furthermore, the results of structure based sequence comparison (http://www.sanger.ac.uk/Software/Pfam/) also indicated that most parts of Rwdd1 and Gir2 might have similar structures (data not shown). Therefore, we predict that Rwdd1 is likely to be another RWD containing IUP.

As predicted by FoldIndex, the C-terminal portion of Rwdd1 had a lower fold score compared with the RWD domain. Consistent with this, we also found that the eukaryote expressed C-terminal portion of Rwdd1 exhibited a bigger molecular mass discrepancy than its prokaryote expressed counterpart when compared with the N-terminal portion, suggesting the eukaryotic modification happens primarily at the C-terminal part of Rwdd1. It was reported that some post-translational modifications, such as phosphorylation, amidation, glycosylation and ubiquitination, were strongly correlated with IUP (12). As a result, we could deduce from the above facts that the C-terminal portion of Rwdd1 might be the major disordered region. It is probably a better target region for post-translational modifications.

IUP may gain much more stabilization energy from intermolecular contacts, than from folding, and are specialized for protein-protein interactions (13). Following binding to its target, IUP may undergo disorder-to-order transition and carry out their regulatory function (8). Rwdd1 is highly acidic. This may be critical for Rwdd1 to remain an unstructured form in cytoplasm before it could interact with certain protein molecule and carry out its function. The structure of the RWD domain of mouse GCN2 had been solved to be of an α-β sandwich form (6). Thus, the RWD region in Rwdd1 might transit from unfolded state to folded state when complexing with a partner. As Rwdd1 lacks certain catalytic domain, it presumably plays a regulatory role *in vivo* by competing with certain RWD containing proteins.

Thymus is a central lymphoid organ where T lymphocytes undergo selection and maturation. The size of the thymus is reduced in aged hosts, with a prominent loss of thymocytes and thymic epithelial cells and a decrease of mature T lymphocyte output (14). As T lymphocytes play essential roles in adaptive immunity, involution of the thymus may be a primary cause for age-associated decrease of T cell immune responses (15, 16). However, due to the complexity of the aging process, the underlying mechanisms of age-related thymus involution are not well understood. To investigate the gene expression patterns in thymuses from aged mice, we performed differential display reverse

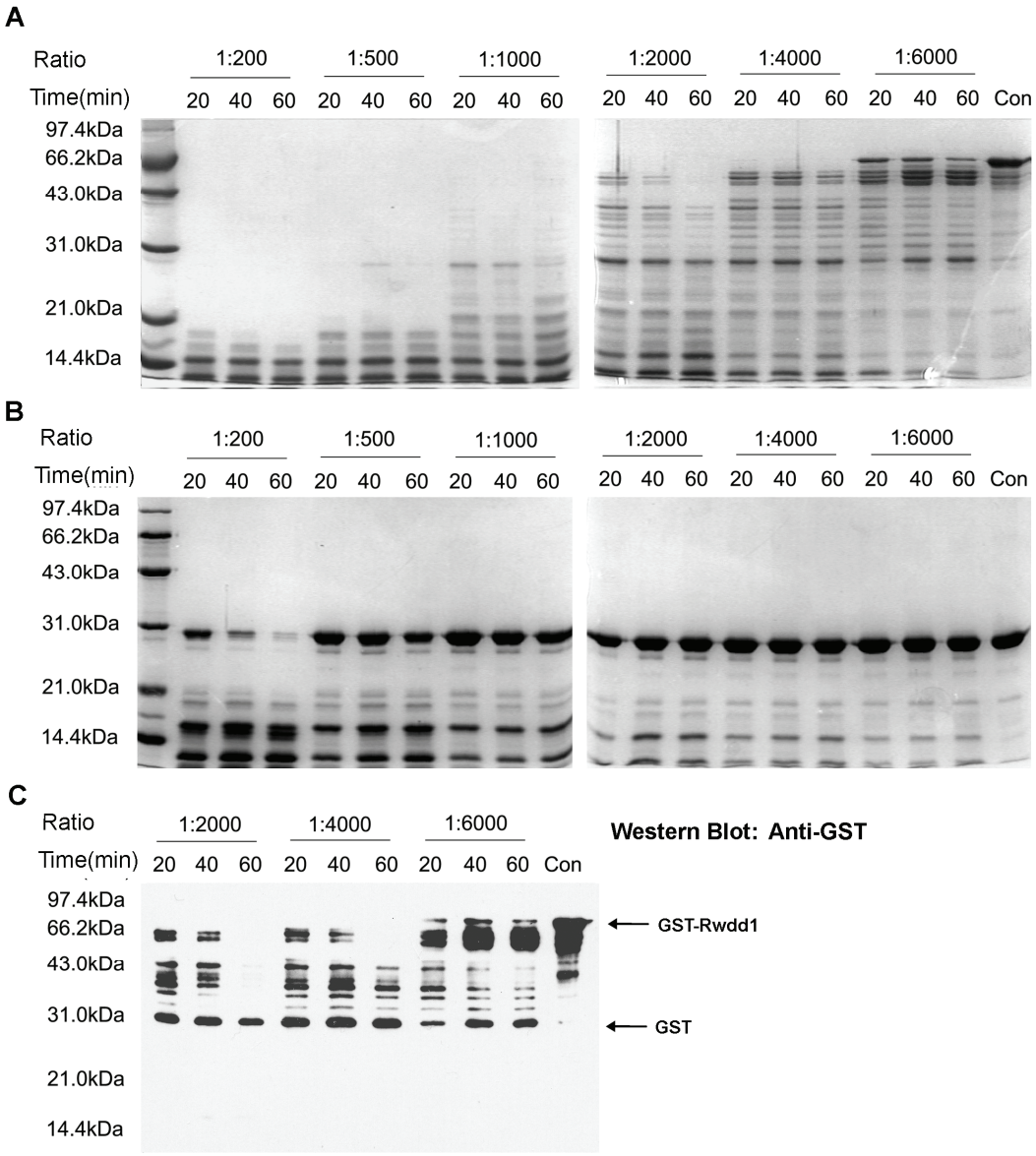

Figure 3. Limited proteolysis of GST-Rwdd1 and GST. (A) Purified GST-Rwdd1 (0.5 mg/ml) was subjected to limited degration by proteinase K under different proteinase to target protein ratios (w/w) for different time period. (B) Purified GST was subjected to degration under the same condition. (C) Western blot of the limited proteolysed GST-Rwdd1 using anti-GST as the primary antibody.

transcriptase polymerase chain reaction (DDRT-PCR) using total RNA from thymus of 1-month and 10-month old mice. Our previous study suggested Rwdd1 is a thymus-aging related protein (1). Currently, the detailed way of Rwdd1 functioning is still unclear. However, the biophysical characteristics of this protein may be helpful in providing some clues for further understanding its role in cellular signal network.

Acknowledgements

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protein that is present in Saccharomyces cerevisiae as a group of heterogeneously electrophoretic migrating forms. Biochem Biophys Res Commun. 2005;332:450-455.
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