Article

Identification and Characterization of a Novel Thymus Aging Related Protein Rwdd1

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By using DDRT-PCR and EST segment ligation, a novel mouse thymus involution related gene *Rwdd1* was identified. The reading frame encoded a protein of 243 amino acid residues which contained an RWD domain at the N terminus. Rwdd1 expression in thymus was decreased in aged and oxidatively stressed mice. It was found to be expressed extensively in thymocytes and thymic epithelial cells. The expression level of Rwdd1 could affect the transactivation activity of androgen receptor (AR) in transiently transfected thymic epithelial cells. However, no direct interaction could be detected by fluorescence resonance energy transfer (FRET) analysis. In conclusion, Rwdd1 is a thymus involution related protein that may indirectly affect AR signaling pathway. *Cellular & Molecular Immunology*. 2008;5(4):279-285.

Key Words: Rwdd1, thymus, aging, androgen receptor

Introduction

Thymus is the organ where immature T cells proliferate and differentiate (1). However, it will undergo a progressive reduction in size during aging, with a profound loss of thymocytes and thymic epithelial cells (2, 3). This “thymus involution” may be due to diminished numbers of T cell precursors, decreased capacity to rearrange T cell receptor (TCR) genes, or alteration of the thymic microenvironment owing to variation of cytokine and hormone levels (2).

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 thymus from aged mice, we performed differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) by using total RNA from thymus of 1-month and 10-month old mice. We cloned the full-length cDNA of a novel gene termed *IH1* (GenBank accession No. AF503942), and found the expression of IH1 was decreased in thymus of aged mice. IH1 was renamed as Rwdd1 based on the RWD domain located at its N-terminus.

The counterpart of Rwdd1 in the rat is also called small androgen receptor interacting protein. Much evidence suggests that androgen receptor (AR) is related to thymus involution: testosterone, ligand of AR, appeared to inhibit thymocyte development; castrated male animal showed apparent thymus enlargement, whereas testosterone treatment could restore the thymus to the original size; furthermore, androgen deprivation could lead to apparent augmentation of T cell levels and responses (4-6).

In the present study, we investigated the relationship between Rwdd1 and thymus aging. At the same time, we studied the role of Rwdd1 in AR signaling pathway.

Materials and Methods

Animals

Male BALB/c (H-2^d) mice, 4 weeks or 40 weeks old, were purchased from the Laboratory Animal Center of the Chinese National Institute for the Control of Pharmaceutical and Biological Products. Mice were housed under specific pathogen free conditions with autoclaved water and controlled feeding until used. Mouse oxidatively stressed model was established as described by Feng et al. (7). Briefly, mice were exposed to ozone at a concentration of 1.2 mg/m^3, 10 h per day for 15 days. Untreated mice were used as controls.

Differential display of mRNA and cloning of full-length cDNA

Thymus was removed from 1-month or 10-month old mice. Total RNA (200 μg) was prepared using Trizol reagent

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Received Mar 28, 2008. Accepted May 26, 2008.

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(Invitrogen) and transcribed to cDNA using oligo dT primers and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. cDNA (1 μl) was added to the PCR mixture containing [α-^{32}P] deoxyadenosine triphosphate (10 mCi/ml), 10-mer deoxyoligonucleotide random 5' primer, and 3'-oligo $dT_{12}N$ (N is A, C or G) single-anchored primer. Amplification was carried out as two stages: stage 1, 40 cycles each consisting for 30 sec at 95°C, 2 min at 40°C, and 2 min at 72°C; stage 2, 10 min at 72°C. The radio labeled DNA fragments were electrophoresed in 6% denaturing polyacrylamide gels, and dried gels were exposed to X-ray films. Differentially expressed DNA fragments were cut from the dried gels and extracted by boiling in 50 μl ddH_2O.

The isolated cDNAs were reamplified and the products were denatured and dotted onto two identical nylon membranes. Reverse dot-blot hybridization was carried out by using radiolabeled cDNAs prepared with total RNA from 1-month and 10-month old mouse thymuses as probes. Those cDNA fragments, whose expression differences had been confirmed were cloned into pGEM-T Easy Vector and sequenced and submitted to GenBank as ESTs.

One of the ESTs was highly expressed in 1-month old mouse thymus. It was homologous to EST aa59668. By searching NCBI-Unigene database, a mouse unigene was found with Unigene ID as Mm.28528.

A pair of primers was designed according to the sequence of Unigene Mm.28528. Forward: 5'-CAC GAT GAC AGA TTA CGG CGA G-3'; reverse: 5'-TCC AAA CCC TCT AGC ATA GCC AC-3'. An 803-bp sequence was obtained by RT-PCR and sequenced. By searching HGMP database (Human Genome Mapping Project, http://www.hgmp.mrc.ac.uk), several homologous ESTs were found, including BG149005, BF467896, BE951684, BE854389, BF468150, BG276500, BG145339, and BF714950. By using Seqman (DNASTAR, WI, USA), the 803 nt sequence was extended to 1,004 nt by ligating these ESTs.

Northern blot analysis

Total RNAs were prepared from thymuses of oxidatively stressed or control mice using the Trizol reagent. Twenty μg of total RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred onto a positively charged nylon membrane (Amersham). Radiolabeled cDNA probes were prepared using random primer labeling kit (Progema) by [α-^{32}P] deoxycytidine triphosphate incorporation. Autoradiography was used to visualize the positive hybridization signals. Blots were stripped and reprobed for β-actin.

Preparation and sorting of thymocytes

Thymocytes were freshly isolated from 4-week old mice. Red blood cells were removed using hypotonic lysis buffer (0.14 M NH_4Cl buffered with 0.017 M Tris, pH 7.2) for 5 min at room temperature. Remaining cells were incubated with saturating amounts of phycoerythrin-conjugated anti-mouse CD8 antibodies and fluorescein isothiocyanate-conjugated anti-mouse CD4 antibodies (BD PharMingen) and sorted with a FACSAria^{TM} Cell Sorter (Becton Dickenson).

Reverse transcriptase polymerase chain reaction (RT-PCR)

The mRNA from cells was prepared using Trizol reagent and transcribed to cDNA using oligo dT primers and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative real-time PCR reaction was performed in a volume of 20 μl containing oligonucleotide primers (5 μM each), and SYBR Green PCR Master Mix (Applied Biosystems) containing Taq DNA Polymerase, the reaction buffer, dNTP and the double strand DNA-specific fluorescent dye SYBR Green. The following primers were used: Rwdd1-fw: 5'-GAA ACT GTT CCA TGG CAC C-3', Rwdd1-rv: 5'-TCC CTG CTT GCT CCT CTT-3'; AR-fw: 5'-GAA GAC CTG CCT GAT CTG TGG-3', AR-rv: 5'-TGT TTC CCT TCA GCG GCT C-3'; Actin-fw: 5'-ACT GTG CCC ATC TAC GAG GG-3', Actin-rv: 5'-GTG GTG GTG AAG CTG TAG CC-3'. Amplification was carried out as two-step procedure: denaturation at 95°C for 10 min and 40 cycles with denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 45 sec. The fluorescent signal from the samples was measured at the end of the elongation step. Results were analyzed with Sequence Detection Software version 1.2 (Applied Biosystems) and reported as relative ratio to CD4 and CD8 double positive (DP) expression level.

Plasmid constructions

The mammalian expression vectors were generated by cloning RT-PCR amplified inserts into plasmid vectors. The primers were: pcDNA3.1-AR: AR-fw, 5'-CGG GAT CCG CTC AAG GAT GGA GGT GCA GTT AG-3', AR-rv, 5'-CCG CTC GAG TCA CTG TGT GTG GAA ATA GAT GGG CTT-3'; pDsRed-C1-AR: Red-AR-fw, 5'-CCG CTC GAG TAC AGA CAA GCT CAA GGA TGG AGG-3', Red-AR-rv, 5'-CGG GAT CCC TGT GTG TGG AAA TAG ATG GGC TT-3'; pcDNA3.1-Rwdd1-V5: Rwdd1-V5-fw, 5'-CGG GAT CCG CCA CGA TGA CAG ATT ACG GC-3', Rwdd1-V5-rv, 5'-CCG CTC GAG GTC TGA GGA GTC ACT CCC TGG-3'; pEGFP-N1-Rwdd1: Rwdd1-GFP-fw, 5'-CCG GAA TTC CGA TGA CAG ATT ACG GCG AG-3', Rwdd1-GFP-rv, 5'-CGC GGA TCC TCT GAG GAG TCA CTC CCT GG-3'.

Reporter plasmid pARE2-TATA-Luc was a kind gift from Dr. Palvimo JJ (University of Helsinki, Finland) (8). Plasmid pRL-TK was purchased from Promega.

Cell culture and transfection

Human embryonic kidney cell line 293T (ATCC CRL-11268™) was maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS) (Gibco). Thymic epithelial cell lines 1308.1, 427.1, and 6.1.7 were kindly provided by Dr. Barbara Knowles (The Jackson Laboratory, Bar Harbor, ME, USA) (9). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. In case of testosterone treatment, phenol red-free RPMI 1640 or DMEM were supplemented with 10% charcoal stripped FBS (Biological Industries). Plasmids were transiently transfected into 293T or 427.1 cells using Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions.

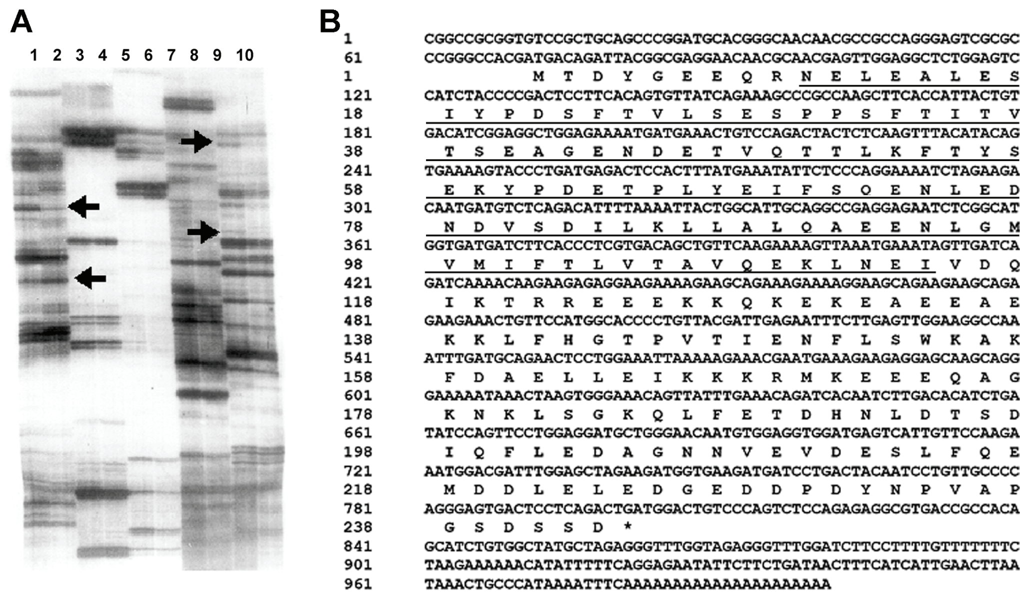

Figure 1. Expression of *Rwdd1* in 1-month old and 10-month old mouse thymuses and its sequence. (A) Differentially expressed gene fragments identified in 1-month and 10-month old mouse thymuses by DDRT-PCR. Lanes 1, 3, 5, 7 and 9, RT-PCR products from 1-month old mouse thymic total RNA; Lanes 2, 4, 6, 8 and 10, RT-PCR products from 10-month old mouse thymic total RNA. Arrows represents differentially expressed gene fragment in certain month-old mice. (B) Nucleotide sequence of *Rwdd1* cDNA and its deduced amino acid sequence. Rwdd1 protein is composed of 243 amino acid residues with an RWD domain at its N-terminus (underlined).

RNA interference (RNAi)

Small interfering RNA (siRNA) specifically targeting mouse *Rwdd1* (5'-GCU UCA CCA UUA CUG UGA CTT-3') was synthesized (Ambion). Transfection of siRNA was carried out according to the manufacturer's protocol using Lipofectamine™ 2000 transfection reagent. RNAi negative control with similar nucleic acid composition was also synthesized by Ambion.

Luciferase reporter assay

To evaluate AR activity, 427.1 cells were seeded on 24-well plate and transfected with 200 ng of pcDNA3.1-AR, 400 ng of pcDNA3.1-V5 or pcDNA3.1-Rwdd1-V5, 400 ng of pARE2-TATA-Luc, and 10 ng of pRL-TK. For RNA interference, 50 pmol of Rwdd1 small interfering RNA (siRNA) or negative control was transfected along with pcDNA3.1-AR, pARE2-TATA-Luc and pRL-TK. Six hours after transfection, cells received fresh medium supplemented with charcoal stripped FBS. Eighteen hours later, cells were treated with 100 nM testosterone for 24 hours. The luciferase activity was assayed and normalized with respect to *Renilla* luciferase activity using Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's guidelines.

Immunofluorescence assay

293T cells, seeded on glass coverslips on 6-well plates, were transfected with Lipofectamine™ 2000 with 1 μg of pEGFP-N1-Rwdd1 and 4 μg of pDsRed-C1-AR. Twenty four hours later, transfected cells were cultured in the absence or presence of 100 nM testosterone for additional 24 h and then used for direct immunofluorescence detection using Leica TCS SP2 laser-scanning microscope. Nuclei were detected by DAPI (Sigma) staining following the manufacturer's instructions.

Fluorescence resonance energy transfer (FRET) assay

293T cells were transiently transfected with pEGFP-N1 or pEGFP-N1-Rwdd1 and pDsRed-C1-AR expression vectors. After 48 h of culture period, FRET was measured using GFP or Rwdd1-GFP as donor and Red-AR as acceptor. The potential of dynamic interaction of Rwdd1-GFP and Red-AR following testosterone treatment was recorded in time series. In brief, cells were cultured in Willco-dish® (WillCo Wells BV) and placed on a 37°C warm stage during FRET assay. Testosterone was added in the culture medium to a final concentration of 1 mM. Then images were taken every 1 min for a total of 30 frames. The Rwdd1-GFP expressing cells were visualized by illumination with 488 nm laser line with the detection window at 500-530 nm. FRET, GFP fluorescence bleed-through and RFP emission resulting from the laser (488 nm) excitation were detected at the FRET channel with the detection window at 560-670 nm. FRET efficiency was calculated using Leica Confocal Software Version 2.00.

Statistics

Student's *t* test was used in the statistical analysis.

Results

Isolation and identification of Rwdd1 gene

By applying DDRT-PCR, 108 cDNA fragments were found

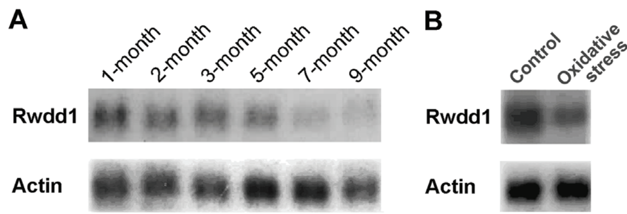

Figure 2. Expression of Rwdd1 in aging and oxidatively stressed mouse thymuses. (A) Northern blot result of Rwdd1 transcripts in mouse thymuses of different ages. Total RNA was isolated from thymuses of 1-, 2-, 3-, 5-, 7- and 9-month old mice and then transferred onto a positively charged nylon membrane. Radiolabeled probe was used to detect Rwdd1 mRNA expression. Hybridization of β-actin was used as an internal control. (B) Comparison of Rwdd1 mRNA expression in oxidatively stressed and control mouse thymuses.

to be differentially expressed in 1-month old and 10-month old mouse thymuses (Figure 1A). Among them, 31 fragments were further confirmed by reverse dot blot hybridization. One of the cDNA fragments was highly expressed in 1-month old mouse thymus. Its full-length cDNA was obtained by EST ligation using tools of bioinformatics, sequenced and assigned the GenBank accession No. AF503942. We named it *IH1*, but now its official name is *Rwdd1*, i.e., RWD domain containing 1. The cDNA of *Rwdd1* consisted of 1,001 bp, containing an open reading frame of 732 bp with 5' and 3' noncoding regions of 70 and 199 bp, respectively. The deduced protein contained 243 amino acid residues and was found to have an RWD domain (residues 10-114) (Figure 1B).

Rwdd1 expression in aging and oxidatively stressed mouse thymus

To determine whether Rwdd1 expression changes with thymus development, total RNAs were prepared from thymuses of mice at different ages and analyzed by Northern blot. The expression level of Rwdd1 was down-regulated during thymus aging (Figure 2A). There was also a significant decrease of Rwdd1 mRNA expression in oxidatively stressed mice (Figure 2B).

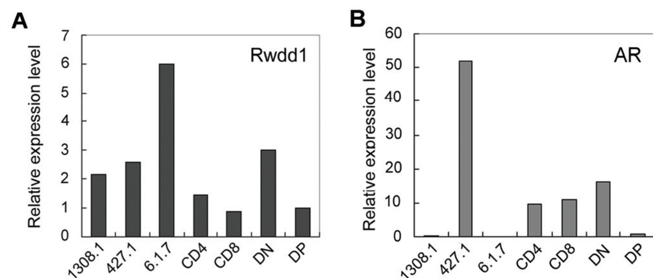

Figure 3. Expression of Rwdd1 and AR in thymocytes and thymic epithelial cell lines. Mouse thymocytes were FACS sorted as $CD4^+$, $CD8^+$, DP, and DN cells. Then RNA was extracted for semi-quantitative realtime PCR analysis of Rwdd1 and AR mRNA levels. Expression of Rwdd1 and AR was also evaluated in three thymic epithelial cell lines, 6.1.7, 427.1, and 1308.1.

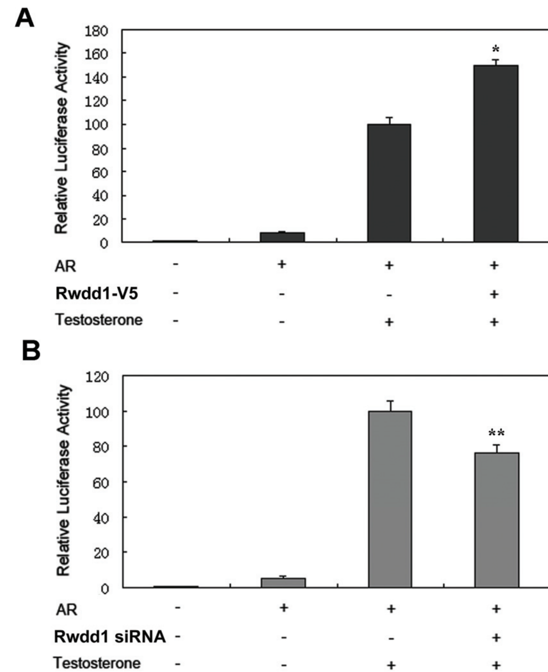

Figure 4. Expression of Rwdd1 and AR transactivation activity. (A) 427.1 cells were transfected with pcDNA3.1-Rwdd1-V5 or pcDNA3.1/V5 together with pcDNA3.1-AR, pARE2-TATA-Luc, and pRL-TK. After 48 h, cells were harvested and assayed for luciferase activity. (B) 427.1 cells were transfected with Rwdd1 siRNA or negative control RNA together with pcDNA3.1-AR, pARE2-TATA-Luc and pRL-TK. After 48 h, cells were harvested and assayed for luciferase activity. The results were corrected by *Renilla* luciferase activity. *$p < 0.01$,** $p < 0.05$

Expression of Rwdd1 and AR in thymocytes and thymus derived cell lines

Thymocytes were FACS sorted as $CD4^+$, $CD8^+$, $CD4^-CD8^-$ (DN), and $CD4^+CD8^+$ (DP) cells. Total RNAs were prepared and reverse transcribed. All the four thymocyte subsets expressed Rwdd1 and AR, among which the highest expression of Rwdd1 and AR appeared in DN thymocytes. Rwdd1 expression in the thymus medulla derived cell line 6.1.7 was higher than those in two thymus cortex derived cell lines, 1308.1 and 427.1. However, only 427.1 cells expressed AR, with the expression level approximately three fold than that of DN thymocytes (Figure 3).

Expression of Rwdd1 could affect AR transactivation activity

In order to study if Rwdd1 could function in AR signaling, AR and Rwdd1 expression plasmid constructs were transfected together with reporter plasmid pARE2-TATA-Luc to 427.1 cells. Plasmid pRL-TK was cotransfected for normalization. As shown in Figure 4A, in the presence of 100 nM of testosterone, overexpression of Rwdd1 enhanced AR-dependent transactivation by 49.8% ($p < 0.01$). In accordance

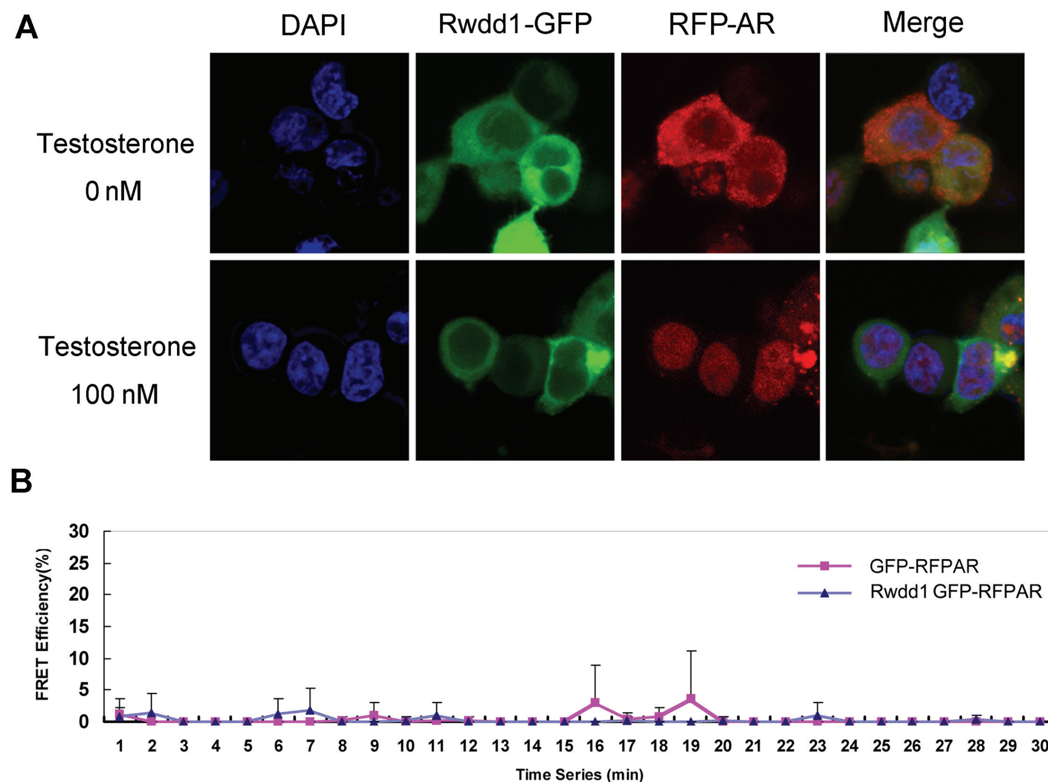

Figure 5. Intracellular localization and FRET assay of Rwdd1 and AR. (A) 293T cells were cotransfected with pEGFP-N1-Rwdd1 and pDsRed-C1-AR. Twenty-four hours after transfection, the culture medium was changed. The cells were incubated for another 24 hours in the absence or presence of 100 nM testosterone. Localization of the fusion proteins was visualized under a confocal microscope. (B) 293T cells were transiently transfected with pDsRed-C1-AR and pEGFP-N1-Rwdd1, or pDsRed-C1-AR and pEGFP as control. Cells were cultured in phenol red free RPMI 1640 medium supplemented with 10% charcoal stripped FBS. Seventy-two hours later, cells were observed under a confocal microscope. FRET efficiency was calculated for thirty minutes with 1 mM testosterone addition as the start point using Leica Confocal Software Version 2.00.

with this result, cotransfection of Rwdd1 siRNA decreased AR-dependent transactivation by 24.1% ($p < 0.05$), as shown in Figure 4B.

Rwdd1 and AR had similar localization in the absence of testosterone

Because the distribution of a potential transcriptional coregulator may give insight into the mechanism of its action, we examined the intracellular localization of Rwdd1 and AR in the absence or presence of testosterone. Rwdd1-GFP and Red-AR had a similar localization in the absence of testosterone. However, upon testosterone exposure, Red-AR fusion protein translocated into the nucleus, while Rwdd1-GFP still stayed in the cytoplasmic compartment (Figure 5A).

FRET analysis did not support the existence of direct interaction between Rwdd1 and AR

FRET, the fluorescence resonance energy transfer, is a distance-dependent interaction between excited fluorescence dye molecules in which the excitation transfer from the donor molecule to the acceptor molecule takes place without emitting photons. 293T cells were cotransfected with pEGFP-N1-Rwdd1 and pDsRed-C1-AR expression vector. The FRET efficiency of the expressed Rwdd1-GFP and Red-AR showed no significant difference to GFP and Red-AR, even following testosterone exposure (Figure 5B).

Discussion

Age-associated alterations of the immune system are important to the health of aging individuals. Older subjects tend to be more susceptible to microbial infections, certain autoimmune diseases and specific cancers (10-12). The thymus is a lymphoid organ that is in charge of T lymphocyte development, selection and maturation. It shows an apparent reduction in size during aging, with a prominent loss of thymocytes and thymic stromal cells and a decrease of T cell output. As T cells perform essential functions in adaptive immunity, the involution of the thymus is closely related to age-associated changes of the immune response (13, 14).

Due to the complexity of the aging process, the underlying mechanisms of age-related thymus involution are not well understood at present. *Rwdd1* was detected by DDRT-PCR using total RNA from thymus of 1-month and 10-month old mice. The expression of Rwdd1 in the thymus was

decreasing with age. Accordingly, Rwdd1 expression was down-regulated in thymus from oxidatively stressed mice, the senescence model resulting from free radical damage (7, 15). Furthermore, the expression level of Rwdd1 was higher in immature DN T cells compared with more mature T cells. These results suggest that Rwdd1 is related to thymus aging. However, what is the molecular mechanism of its function?

It should be noted that in mammalians, for instance, in humans, a significant reduction of thymic mass starts at the age of one year and results in an important reduction of thymic mass by the time of puberty (16). As a consequence, it is deduced that hormones may induce, or at least influence age-associated thymic loss. Lots of evidence suggested that androgen might induce thymus involution by inhibiting thymocyte development and accelerating T cell apoptosis *via* AR expressed in the thymus (17-23). Furthermore, several studies suggested that AR in the thymic epithelial part might be responsible for the androgen induced thymic involution (24-26). However, mechanisms of androgen-induced thymic involution remain largely unclear.

Rwdd1 is the abbreviation of RWD containing 1. RWD is a domain named after three major RWD containing proteins: RING finger-containing proteins, WD domain-containing proteins and DEAD-like helicases (27). However, its function is not fully understood yet. The counterpart of Rwdd1 in the rat, which showed 99.2% similarity in the amino acid sequence with the murine one, is also called small androgen receptor interacting protein (data from RGD-Rat Genome Database). Therefore, we imagined that Rwdd1 might be a potential AR coregulator and studied the relationship between them. We used thymic epithelial cell line 427.1 to study the relationship because it had high level of Rwdd1 and AR expressions simultaneously. Interestingly, Rwdd1 could enhance the transactivation activity of AR, whereas no direct interaction between these two proteins was found by intracellular localization observation and FRET analysis. Even between Rwdd1 and testosterone bound AR, there was not a direct interaction existing.

Classical AR belongs to nuclear receptor superfamily. Recently, there are an increasing number of AR coregulators identified. These coregulators use multiple mechanisms to influence AR transcription. They modulate in: remodeling or recruitment of general transcription factors; phosphorylation or ubiquitination of AR; appropriate folding of AR; ligand binding capability of AR; nuclear translocation of AR; and N- and C-terminus interaction of AR (28, 29). Currently, we are not sure of the exact mechanism by which Rwdd1 affects AR signaling. However, undoubtedly such modulation of AR activity might be accomplished in the cytoplasm, since Rwdd1 would not migrate with AR to the nucleus upon testosterone treatment.

In conclusion, Rwdd1 expression is decreasing during thymus aging. Till now we are not sure whether it is the cause or the result of the involution process. Although Rwdd1 may influence AR signaling indirectly, it is apparently not the only reason to explain the complicated thymus aging process. The entire phenomenon deserves further research.

Acknowledgements

We would like to thank Dr. Barbara Knowles for supplying the thymic epithelial cell lines, and Dr. Jorma. J. Palvimo for providing the reporter plasmid. This work was supported by a grant (JaICA 2000-2003) from the Japan Institute for the Control of Aging and the funds from the National Natural Science Foundation of China (No. 30471589) to Dr. Wei He.

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