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Ubiquitin-specific protease activity of *USP9Y*, a male infertility gene on the Y chromosome

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Abstract. Deletions of *USP9Y* have been observed among infertile males with defective spermatogenesis. Therefore, the gene has been designated as a male infertility gene on the Y chromosome. However, it remains to be determined how male infertility results from deletions of this gene. In order to initiate an investigation into the cellular functions of USP9Y in male germ cell development, in the present study we characterized the enzymatic specificity of USP9Y. Our results show that both USP9Y and Fam, the mouse infertility protein Usp9x, possess a protease activity specific to ubiquitin. These results suggest that, through de-ubiquitination, USP9Y may stabilize a specific target protein that is important for male germ cell development.

Extra keywords: Fam, male infertility.

Introduction

Male infertility accounts for approximately half the cases of couple infertility and a significant proportion of male infertility can be ascribed to a genetic aetiology (Liford *et al.* 1994). Microdeletions at specific loci of the long arm of the human Y chromosome are frequently detected in azoospermic and oligozoospermic patients (Najmabadi *et al.* 1996; Qureshi *et al.* 1996; Vogt *et al.* 1996; Foresta *et al.* 1997; Girardi *et al.* 1997; Kremer *et al.* 1997; Pryor *et al.* 1997; Simoni *et al.* 1997; van der Ven *et al.* 1997). At least three regions of Yq have been defined as *AZF* loci (*AZFa*, *AZFb* and *AZFc*) and the genes at these loci have been proposed as candidate genes for male infertility (Vogt *et al.* 1992, 1996; Pryor *et al.* 1997; Elliot *et al.* 1997). However, it remains to be determined how male infertility results from deletions of these genes.

USP9Y is a gene located at *AZFa*. A point mutation of *USP9Y* results in partial arrest of spermatogenesis at the spermatocyte stage with few post-meiotic germ cells (Sun *et al.* 1999). *USP9Y* encodes a large polypeptide of 2555 amino acids that is approximately 300 kDa in size (Brown *et al.* 1998). The polypeptide does not contain known functional domains except for the Cys and His domains, which are characteristically present in ubiquitin-specific proteases (UBPs; Baker *et al.* 1992).

The enzymatic characteristic of UBP is to remove ubiquitin from conjugated proteins or from poly-ubiquitin tails (Tobias and Varshavsky 1991; Baker *et al.* 1992; Papa *et al.* 1993; Wilkinson *et al.* 1995). In order to initiate an investigation into the cellular functions of USP9Y in male germ cell development, we wanted to confirm the enzymatic activity that USP9Y possesses. In the present study, the UBP activity of human USP9Y was confirmed using an *Escherichia coli*-based assay system, as well as a mammalian tissue culture system.

Materials and methods

Cloning the Cys and His domains of the human USP9Y *gene*

Because the *USP9Y* gene, which is 2555 amino acids long, is too large to handle, we decided to use the Cys and His domains of USP9Y (U9CHD) for UBP assays. *U9CHD* was polymerase chain reaction (PCR) amplified from first-stranded cDNA of human testis tissue with a specific primer set (forward primer: 5′-CGTCACCCGTTACCATCAAT-3′; reverse primer: 5′-CGCATCTATCATATCCATTTGTTCA-3′). The PCR cycle was composed of 40 cycles of 94°C for 40 sec, 55°C for 40 sec, and 72°C for 2 min. The PCR fragment of *U9CHD* (1402 bp) was subcloned into the *pGEM-T* vector (Promega, Madison, WI, USA) and its sequence was confirmed. *U9CHD* was further subcloned into the *pGEX-4T-1* vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for bacterial expression or into the *pCMV-Tag3B* vector (Stratagene, La Jolla, CA, USA) for mammalian cell expression. For detection of the U9CHD protein, the Myc epitope was tagged at the N-terminal end.

The mouse *Fam* cDNA was a generous gift from Dr Stephen A. Wood (University of Queensland, Brisbane, Qld, Australia). The full-length *Fam* cDNA was subcloned into *pGEM-4T-1* for bacterial expression.

Bacterial transformation for the UBP assay

The *JM101* strain of *E. coli* that expresses either Ub-R-βGal or Ub-M-βGal was transformed with *pU9CHD*, *pFam* or *pHAUSP* (herpes

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virus-associated ubiquitin-specific protease; Everett *et al.* 1997) and plated onto LB agar with 100 μg mL^{-1} amphicillin, 34 μg mL^{-1} chloramphenicol, 40 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG). Plates were incubated at 37°C for 18 h and their colony colours were checked.

Ubiquitin-specific protease activity assay in mammalian cells

The expression vector for *U9CHD* or *HAUSP* was cotransfected into COS7 cells with the construct for hemagglutinin (HA)-tagged ubiquitin or Myc-tagged NEDD8. Sixteen hours after transfection, cells were treated with 25 μM calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal (ALLN); Sigma, St Louis, MO, USA) and cultured for a further 10 h. Cells were washed twice with phosphate-buffered saline (PBS), dissolved into the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to immunoblot analysis using antibodies against the HA tag (Babco, Berkeley, CA, USA) or the Myc tag.

Immunoblot analysis

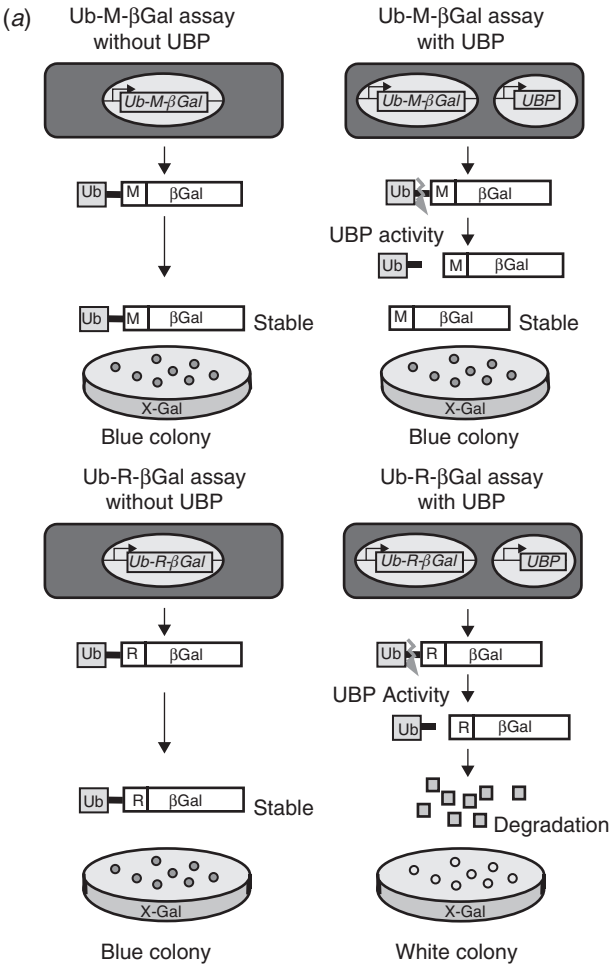
Proteins from harvested cells were fractionated with a 10% SDS–polyacrylamide gel for detection of the ubiquitin- or NEDD8-conjugated proteins or with a 15% gel for the U9CHD or USP69 proteins. After electrophoresis, protein samples were transferred onto nitrocellulose membranes. The membranes were blocked for 1 h in Blotto (1 × TBS (Tris-buffered saline), 0.1% Tween 20, 5% non-fat dried milk), incubated with the primary antibody for 1 h, washed with 1 × TBST (Tris-buffered saline, 0.1% Tween 20) three times, incubated with the secondary antibody for 30 min and washed again, three times, with 1 × TBST. Specific signals were detected either with the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) or with the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/*p*-nitro blue tetrazolium chloride (NBT) system (Promega). An antibody specific to the HA tag (1 : 1000 dilution) was used for detection of ubiquitin-HA conjugated proteins. An antibody specific to the Myc tag (1 : 100 dilution) was used for detection of Myc-NEDD8 conjugated proteins and for the detection of the Myc-U9CHD protein.

Results

Escherichia coli-*based* in vivo *assay for UBP activity*

The UBP activity of USP9Y was first examined with the *E. coli*-based *in vivo* assay, illustrated in Fig. 1*a* (Tobias and Varshavsky 1991). This assay method takes advantage of the absence of a ubiquitin degradation system in *E. coli*. The β-galactosidase protein linked to the ubiquitin domain at the *N*-terminal end (Ub-R-βGal or Ub-M-βGal) is stable in bacteria and, as a result, can make colonies blue in X-gal-containing plates. If a *UBP* gene was cotransformed, the ubiquitin domain would be removed from the *N*-terminal end of the fusion protein, leaving β-galactosidase by itself. If the resulting β-galactosidase sequence started from methionine (M-βGal), the protein should be stable enough to make the colonies blue in the presence of X-gal. However, if the β-galactosidase started from arginine (R-βGal), the protein would be unstable and, as a result, the colony colour would remain white.

Cotransformation of *HAUSP*, a positive control gene, with *pUb-R-βGal* resulted in white colonies, whereas cotransformation with *pUb-M-βGal* produced blue colonies (Fig. 1*b*), indicating that our experimental system worked as expected. Under such conditions, we cotransformed *Fam*, the mouse *Usp9x* gene (Kanai-Azuma *et al.* 2000), along with *pUb-R-βGal*, and observed the formation of white colonies (Fig. 1*b*).



(b)

	Ub-M-βGal	U-R-βGalb	UBP Activity
Vector			–
HAUSP			+++
Fam			++
U9CHD			++

Fig. 1. *Escherichia coli*-based *in vivo* assay for ubiquitin-specific protease (UBP) activity. (*a*) Schematic illustration of the *E. coli*-based *in vivo* assay for UBP activity. (*b*) The UBP activity assay for USP9Y and Fam. The expression vector *pGEX-4T-1* was used as a negative control and herpes virus-associated ubiquitin-specific protease (HAUSP) was used as a positive control. Both Fam and U9CHD showed UBP activity in this assay system. Ub-M-βGal, Ub-R-βGal, β-galactosidase protein linked to the ubiquitin domain at the *N*-terminal end, with resulting β-galactosidase sequence starting from methionine or arginine, respectively; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

The UBP activity of Fam appeared to be not as strong as that of HAUSP, but was sufficient enough to produce white colonies. Cotransformation of *U9CHD*, a *USP9Y* fragment with the His and Cys domain, along with *pUb-R-βGal* also produced white colonies, indicating that human USP9Y possesses UBP activity. When *U9CHD* or *Fam* were cotransformed with *pUb-M-βGal*, the colonies turned blue, as expected.

Ubiquitin-specific protease activity assay in mammalian cells

The UBP activity of USP9Y was tested in a mammalian cell system. Treatment of cultured cells with a proteasome inhibitor such as ALLN induces a marked increase in cellular proteins conjugated with ubiquitin, which can be detected as a characteristic ladder-like pattern in immunoblot analysis with an antibody against ubiquitin (Taya *et al.* 1998; Schubert *et al.* 2000). In our experimental system, an HA-tagged ubiquitin expression vector was transfected into COS7 cells for the detection of ubiquitin-conjugated proteins (Fig. 2*a*, lane 3). When a ubiquitin-specific protease gene such as *USP69* (Park *et al.* 2002) was cotransfected into the cell, ubiquitin started to become dissociated from conjugated proteins, resulting in the disappearance of the ladder-like pattern (Fig. 2*a*, lane 5). Under such conditions, cotransfection of *U9CHD* into COS7 cells resulted in disappearance of the ladder-like pattern (Fig. 2*a*, lane 4). These results indicate that USP9Y possesses UBP activity *in vivo*. Expression of Myc-U9CHD and Myc-USP69 proteins was confirmed with an antibody against the Myc epitope (Fig. 2*b*).

USP9Y does not possess NEDD8-specific protease activity

A number of ubiquitin-like proteins have been described, expanding the scope of post-translational protein modification (Baarends *et al.* 2000). For example, NEDD8 shares a strong structural homology with ubiquitin and forms conjugates to their respective targets through a cascade of E1-, E2- and E3-related enzymes (Yeh *et al.* 2000). It is generally assumed that deconjugating enzymes are specific to a single species of the ubiquitin or ubiquitin-like protein. However, a recent report showed that USP21 possesses a dual specificity for both ubiquitin and NEDD8 (Gong *et al.* 2000). Therefore, we decided to examine whether USP9Y also possesses dual specificity.

When Myc-tagged NEDD8 was expressed in COS7 cells, NEDD8-conjugated proteins were detected as a ladder-like pattern in immunoblot analysis (Fig. 3*a*, lane 2). However, ectopic coexpression of U9CHD did not remove the ladder-like pattern of NEDD8-conjugated proteins (Fig. 3*a*, lane 3). These results suggest that USP9Y does not possess NEDD8-specific protease activity. However, we cannot preclude the possibility that an intact USP9Y may possess NEDD8-specific protease activity. Immunoblot analysis with the Myc antibody confirmed that the U9CHD protein was expressed in COS7 cells (Fig. 3*b*).

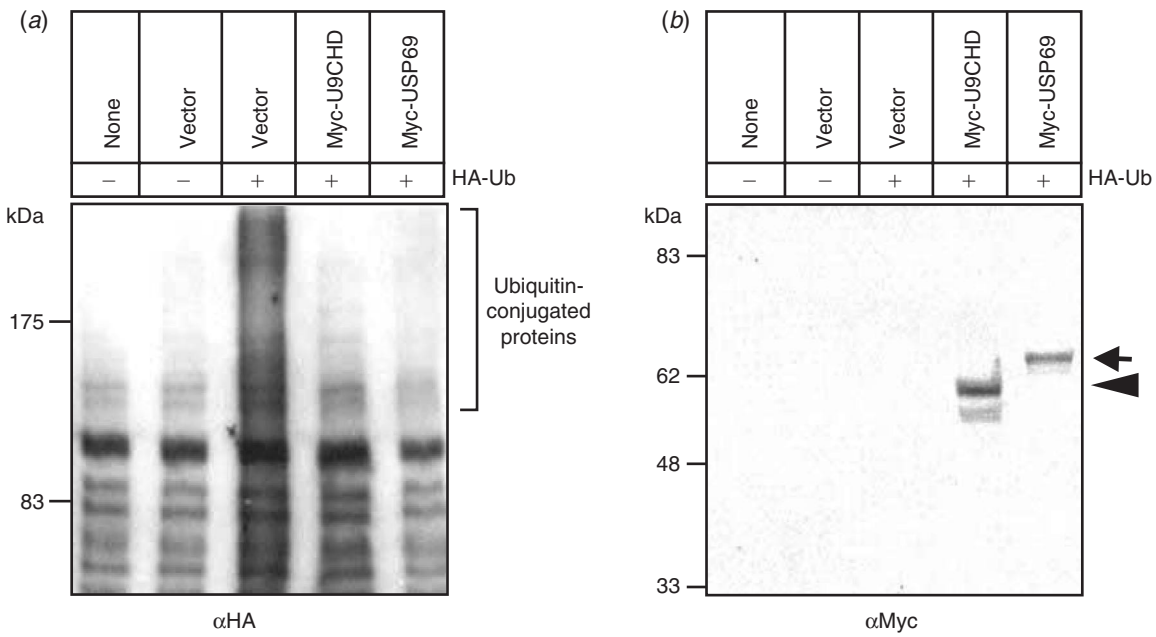


Fig. 2. The ubiquitin-specific protease (UBP) activity assay in COS7 cells. The plasmid for HA-ubiquitin (*pHA-Ub*) was cotransfected with *pMyc-U9CHD* or *pMyc-USP69* into COS7 cells. Total cell lysates were prepared 10 h after *N*-acetyl-Leu-Leu-norleucinal (ALLN) treatment and used for immunoblot analyses with antibodies specific to the HA epitope for detection of (*a*) ubiquitin-conjugated proteins or (*b*) to the Myc epitope for detection of Myc-U9CHD (arrowhead) and Myc-USP69 (arrow) proteins. The Myc vector (*pCMV-Tag3B*) was cotransfected as a control. The same experiments were repeated three times.

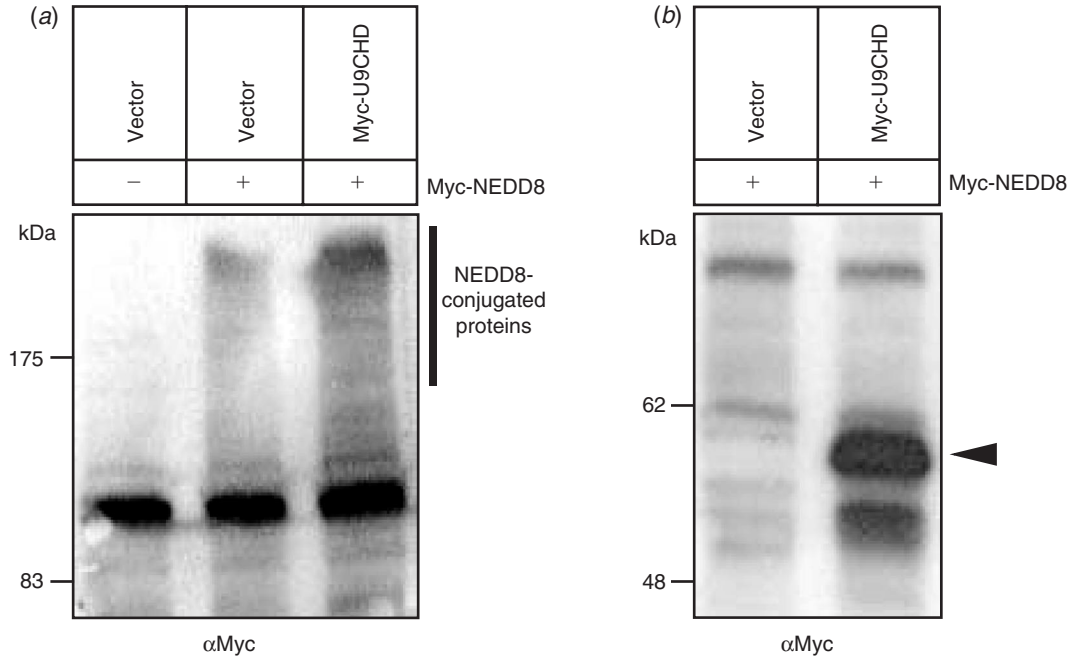


Fig. 3. No NEDD8-specific protease activity in U9CHD. The plasmid for Myc-NEDD8 (*pMyc-NEDD8*) was cotransfected with *pMyc-U9CHD* into COS7 cells. Total cell lysates were prepared 10 h after *N*-acetyl-Leu-Leu-norleucinal (ALLN) treatment and used for immunoblot analyses with the Myc antibody for detection of (*a*) NEDD8-conjugated proteins or (*b*) Myc-U9CHD (arrowhead). The Myc vector (*pCMV-Tag3B*) was cotransfected as a control.

Discussion

In the present study, we confirmed the UBP activity of U9CHD, the Cys and His domain of the human USP9Y protein. Even though the full-length USP9Y protein could not be used in the present study, it is most likely that the protein does possess UBP activity, like Fam (Taya *et al.* 1998). The protease activity of USP9Y seems specific to ubiquitin, at least in that it does not react to NEDD8.

The UBPs comprise a large family, but their biological significance has not yet been fully explored (Chung and Baek 1999). Genetic and biochemical evidence has revealed that UBPs are involved in diverse cellular functions, such as cell growth and oncogenesis, animal development and neuronal function (Hershko and Ciechanover 1998). For example, the *Drosophila fat facet* mutants (*faf*) have defects in embryogenesis as well as in gonad development (Fischer-Vize *et al.* 1992). Because the *faf* phenotype can be suppressed by certain proteasome mutants, it has been proposed that Faf is normally involved in de-ubiquitination of a short-lived key regulatory molecule at specific stages of development (Cadavid *et al.* 2000).

The importance of Fam, a mouse homologue of *Drosophila* Faf, in developmental processes has also been reported. Specific expression of *Fam* during eye development has been observed (Kanai-Azuma *et al.* 2000). Suppression of *Fam* expression in fertilized mouse oocytes resulted in a decrease in cleavage rate and an inhibition of cell adhesive events, suggesting that Fam is required for successful early embryogenesis (Pantaleon *et al.* 2001). Suppression of *Fam* expression was accompanied by a reduction in cellular concentrations of AF-6 and β-catenin (Pantaleon *et al.* 2001), which are believed to be substrates of Fam (Taya *et al.* 1998, 1999).

The results of the present study suggest that, through de-ubiquitination, USP9Y may stabilize specific target proteins that are important for male germ cell development. Obviously, it is critical to identify specific substrates of USP9Y. Initial candidates may be AF-6 and β-catenin, as potential physiological substrates of Fam (Taya *et al.* 1998, 1999).

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