Characterization of alternatively spliced products and tissuespecific isoforms of USP28 and USP25

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Abstract

Background: The ubiquitin-dependent protein degradation pathway is essential for the proteolysis of intracellular proteins and peptides. Deubiquitinating enzymes constitute a complex protein family involved in a multitude of cellular processes. The ubiquitin-specific proteases (UBP) are a group of enzymes whose predicted function is to reverse the ubiquitinating reaction by removing ubiquitin from a large variety of substrates. We have lately reported the characterization of human *USP25*, a specific-ubiquitin protease gene at 21q11.2, with a specific pattern of expression in murine fetal brains and adult testis.

Results: Database homology searches at the DNA and protein levels and cDNA library screenings led to the identification of a new UBP member in the human genome, named USP28, at 11q23. This novel gene showed preferential expression in heart and muscle. Moreover, cDNA, expressed sequence tag and RT-PCR analyses provided evidence for alternatively spliced products and tissue-specific isoforms. Concerning function, USP25 overexpression in Down syndrome fetal brains was shown by real-time PCR.

Conclusions: On the basis of the genomic and protein sequence as well as the functional data, *USP28* and *USP25* establish a new subfamily of deubiquitinating enzymes. Both genes have alternatively spliced exons that could generate protein isoforms with distinct tissue-specific activity. The overexpression of *USP25* in Down syndrome fetal brains supports the gene-dosage effects suggested for other UBP members related to aneuploidy syndromes.

Background

Ubiquitin modification of protein substrates has a major role in a variety of cellular processes such as cell-cycle progression, DNA repair, antigen presentation, differentiation and development, transcriptional activation and selective degradation of damaged proteins (for review see [1-3]). Covalent attachment of ubiquitin molecules is the first step for degradation of the tagged protein via the 26S proteasome pathway. This is a finely regulated and highly specific process as an error in substrate recognition might compromise cell survival.

Studies on the specificity and regulation of ubiquitin-dependent protein degradation have mainly focused on the ubiquitinating enzymes. Nonetheless, there is increasing evidence that other enzymes that remove ubiquitin from the ubiquitin-conjugates (deubiquitinating enzymes, DUBs) not only affect the fate and degradation of intracellular proteins but seem to be essential in the maintenance of cell-free ubiquitin pools [3-5]. Failures of the ubiquitin system have been implicated in many human diseases, among them some important neurodegenerative disorders and several carcinomas. The accumulation of ubiquitin adducts has been described in patients with Alzheimer's, Huntington's, and Parkinson's diseases although the direct involvement of ubiquitinated protein aggregates in the pathological condition has not been proved. On the other hand, a missense mutation in the ubiquitin carboxy-terminal hydrolase L1 (UCHL1) has been found in a German family with Parkinson's disease [6].

Two classes of deubiquitinating enzymes have been described: the UCHs (ubiquitin carboxy-terminal hydrolases) and the UBPs (ubiquitin processing proteases) [4,7,8]. The UCH family members share a small size, cleave ubiquitin from small peptides and amino acids and appear well conserved (40%) across species. In contrast, the UBP family gathers larger and distantly related enzymes that release ubiquitin from a wide range of ubiquitin-protein conjugates [5]. As the human UBP family is likely to be quite large, a systematic nomenclature has been proposed for these enzymes based on the abbreviation USP, for ubiquitin-specific protease [9].

In the UBP family, protein sequence comparisons have shown that homology is restricted to the regions encompassing the active site cysteine and histidine residues and other peptide segments putatively involved in catalysis or substrate binding [7,10]. More than 90 deubiquitinating enzymes have been characterized after data from genome sequencing projects [5]; as many as 16 are encoded in yeast, which is in agreement with the high specificity attributed to this family [2,3]. Although at present the overall homology among UBPs is low, subfamilies of closely related members (60% to 88% amino acid identities) begin to emerge [11-13].

Lately, we have identified a novel UBP member, named *USP25*, in the gene-poor region of human chromosome 21q11.2 and characterized the full cDNA sequence [14]. Isolation of the mouse homolog cDNA allowed us to perform northern and *in situ* hybridization analyses. Higher levels of *USP25* expression in mouse were detected in the proliferative compartments of fetal brain and in maturating spermatocytes of adult testis, allowing the correlation of gene expression with high protein turnover.

Here we describe a human *USP25* homolog, *USP28*, which maps to 11q23 and is preferentially expressed in heart and muscle. Tissue-specific alternatively spliced exons of *USP25* and *USP28* have been identified, in agreement with several isoforms described for vertebrate UBPs [13,15]. The functional deubiquitinating assay has been performed for the two

members of the newly described UBP subfamily. Overexpression of USP25 as measured by real-time PCR in Down versus control fetal brains supports the gene-dosage effects reported for other UBP members.

Results Cloning of USP28

A TBlastN comparison of USP25 sequence against nr database at the National Center for Biotechnology Information (NCBI) server revealed a homologous USP sequence on PAC clone pDJ356d6 (GenBank accession number AC002036) located at 11q23. Specific probes designed following this genomic sequence were used to screen 106 recombinant phages of a human fetal brain cDNA library (Clontech). Six positive clones were isolated, subcloned and sequenced. Four clone sequences were chimeric and were thus discarded. Two overlapping cDNA clones, 5A11 (2.3 kb) and 3A11 (1.5 kb), showed an exact match with chromosome 11 PAC clone pDJ356d6. Evidence that clones 5A11 and 3A11 did not encode the full-length cDNA was based on the protein alignment between the deduced amino acid sequence and USP25, the mRNA size obtained from northern analysis when probed with cDNA clone 5A11, and the absence of the strictly conserved UBP cysteine domain in either clone 5A11 or 3A11.

Three different strategies were followed to isolate the 5' end of *USP28* cDNA. First, a chromosome 11-specific forward primer (5.2 cr11F) was designed from the TBlastN analysis of *USP25* (Figure 1). This sequence corresponded to the 5'-terminal sequence of the predicted exon encoding the cysteine domain in the chromosome 11 PAC DNA. A reverse oligonucleotide, 11race4, was also designed on the basis of the 3A11 cDNA clone sequence (Figure 1). An RT-PCR reaction using heart mRNA as template was subsequently performed with the forward and reverse primers. The expected 958 bp amplified fragment was subcloned in pUC18. Its sequence overlapped with 3A11 and 5A11 cDNA clones, thus allowing the characterization of an additional 908 bp. The overall cDNA sequence, although still incomplete, contained the cysteine domain, together with all other UBP reported signatures.

Second, 10⁶ recombinant phages from kidney and placenta cDNA libraries (Clontech) were screened using three different 5'-*USP28*-specific probes obtained after PCR amplification with primers deduced from the TBlastN comparison to USP_{25} (Figure 1). Five different positive clones were isolated: four from kidney and one from placenta. All inserts were subcloned in pBLUESCRIPT SK+ (Stratagene). Sequence analysis revealed a further 318 bp, although the cDNA was still incomplete.

Third, a 5' RACE experiment was performed on 10^9 phages from the kidney cDNA library with two specific reverse primers (11race6 and 11race7) (Figure 1) and two λ gt10

M T A E L Q Q D D A A G A A D G H G S S 20	Q V P Y R L H A V L V H E G Q A N A G H 600
TGCCAAATGCTGTTAAATCAACTGAGAGAAATCACAGGCATTCAGGACCCTTCCTT	TATTGGGCCTATATCTATAATCAACCCCGACAGAGCTGGCTCAAGTACAATGACATCTCT 1860 Y W A Y I Y N Q P R Q S W L K Y N D I S 620
CATGAAGCTCTGAAGGCCAGTAATGGTGACATTACTCAGGCAGTCAGCCTTCTCACTGAT 180 H E A L K A S N G D I T Q A V S L L T D 60	GTTACTGAATCTTCCTGGGAAGAAGTTGAAAGAGATTCCTATGGAGGCCTGAGAAATGTT 1920 V T E S S W E E V E R D S Y G G L R N V 640
GAGAGAGTTAAGGAGCCCAGTCAAGACACTGTTGCTACAGAACCATCTGAAGTAGGGG 240 E R V K E P S Q D T V A T E P S E V E G 80	AGTGCTTACTGTCTGATGTACATAAAAAAACTACCCTACTCCAATGCAGAGGCAGGC
	CCAACTGAATCAGATGTAGAAGTGGAAGCCCTATCTGTGGAACTCAAGCATTAC 2040 P T E S D Q M S E V E A L S V E L K H Y 680
GATCTTCAGGCTGCCATTGCTTTGAGTCTACTGGAGTCTCCCAAAATTCAAGCTGATGGA 360 D L Q A A I A L S L L E S P K I Q A D G 120	ATTCAGGAGGATAACTGGCGGTTTGAGCAGGAGAGGAGA
5.2.C11F AGAGATCTTAACAGGATGCATGAAGCAACCTCTGCAGAAACGCTCAAAGAGAAAA 420 R D L N R M H E A T S A E T K R S K R K140	TGCAAAATCCCTCAAATGGAGTCCTCCCACCAACTCCTCACAGGACTACTCTACATCA 2160 C K I P Q M E S S T N S S S Q D Y S T S 720
CGCTGTGAAGTCTGGGGAGAAAACCCCCAATGACTGAGGAGAGTTGATGGTTGG 480 R C E V W G E N P N P N D W R R V D G W 160	CAAGAGCCTTCAGTAGCCTCTTCTCATCGGGGTTCGCTGCTTGTCATCTGAGCATGCTGTG 2220 Q E P S V A S S H G V R C L S S E H A V 740
	ATTGTAAAGGAGCAAACTGCCCAGGCTATTGCAAAACACAGCCCGTGCCTATGAGAAGAGGC 2280 I V K E Q T A Q A I A N T A R A Y E K S 760
TTTCAATTGCCTGAATTTCGAAGACTTGTTCTCAGTTATAGTCTGCCACAAAATGTACTT 600 F Q L P E F R R L V L S Y S L P Q N V L 200	GGTGTAGAAGCGGCACTGAGGGGGAGGAGGGGTCATCCTG 2340 G V E A A L S E V M L S P A M Q G V I L 780
GAAAATTGTCGAAGTCATTACAGAAAAGAGAAATATCATGTTTATGCAAGAGGTTCAGTAA 660 E N C R S H T E K R N I M F M Q E L Q Y 220	GCCATAGCTAAAGCCCGTCAGAGCCTTTGACCGAGATGGGTCTGAAGCAGGGCTGATTAAG 2400 A I A K A R Q T F D R D G S E A G L I K 800
TTGTTTGCTCTAATGATGGGATCAAATAGAAAATTTGTAGACCCGTCTGCAGCCCTGGAT 720 L F A L M M G S N R K F V D P S A A L D 240	GCATTCCATGAAGAATACTCCAGGCTCTATCAGCTTGCCAAAGAGACCCCCACCTCTCAC 2460 A F H E E Y S R L Y Q L A K E T P T S H 820
CTATTAAAGGAGCATTCCGATCATCTGAGGACAGCAGCAGCAGGAGATGTGAGTAATTCACA 780 L L K G A F R S S E E Q Q Q D V S E F T 260	AGTGATCCTCGACTTCAGCATGTCCTTGTCTACTTTTTCCAAAATGAAGCACCCAAAAGG 2520 S D P R L Q H V L V Y F F Q N E A P K R 840
CACAAGCTCCTGGATTGGCTAGAGGACGCATTCCAGCTGGCTG	GTAGTAGAACGAACCCTTCTGGAACAGTTTGCAGATAAAAATCTTAGCTATGATGAAAGA 2580 V V E R T L L E Q F A D K N L S Y D E R 860
AGGAACAAATCTGAAAAATCCAATGGTGCAGCTGTTCTATGGTACTTTCCTGACTGA	TCAATCAGCATTATGAAGGTGGCCCAAGCGAAACTGAAGGAAATTGGTCCAGATGACATG 2640 S I S I M K V A Q A K L K E I G P D D M 880
5.1 cr11F \longrightarrow GTCGTGAAGGAAAACCCTTTGTAACGAGACCTTCGGCCAGTATCCTCTTCAGGTA 960 V R E G K P F C N N E T F G Q Y P L Q V 320	AATATGGAAGAGTACAAGAAGTGGCATGAAGATTATAGTTTGTTCCGAAAAGTGTCTGTG 2700 N M E E Y K K W H E D Y S L F R K V S V 900
AACGGTTATCGCAACTTAGACGAGGTGTTTGGAAGGGGCCATGGTGGAGGGGGGATGTTGAG 1020 N G Y R N L D E C L E G A W V E G D V E 340	TATCTCCTAACAGGCCTAGAACTCTATCAAAAGGAAAGTACCAAGAGGCACTTTCCTAC 2760 Y L L T G L E L Y Q K G K Y Q E A L S Y 920
CTTCTTCCCTCCGATCATCGGTAGAGTATGGACAAGGGGGTGGGGTTACCAAAGCTACCT 1080 L L P S D H S V K Y G Q E R W F T K L P 360	CTGGTATATGCCTACCAGAGCAATGCTGCCCGCGGGGGGCCCCGCGGGGGGGCCC2820 L V Y A Y Q S N A A L L M K G P R R G V 940
$ \begin{array}{cccc} CCAGTGTTGACCTTTGAACTCTCAAGATTTGAGTTTAATCAGTCCCTTGGGCAGCCAGAG 1140 \\ P & V & L & T & F & E & L & S & R & F & E & F & N & Q & S & L & G & Q & P & E 380 \end{array} $	AAAGAATCCGTGATTGCTTTATACCGAAGAAAATGCCTTCTGGAGCTGAATGCCAAAGCA 2880 K E S V I A L Y R R K C L L E L N A K A 960 -5A11.1
AAAATTCACAATAAGCTGGAATTTCCCCAGATTATTTATATGGACAGGTACATGTACAGG 1200 K I H N K L E F P Q I I Y M D R Y M Y R 400	GCTTCTCTTTTTGAAACAAATGATGATCACTCCGTAACTGAGGGCATTAATGTGATGAAT 2940 A S L F E T N D D H S V T E G I N V M N 980
AGCAAGGAGCTTATTCGAAATAAGAGAGAGTGTTATTCGAAGGTGGAAGGAGAAATAAAA 1260 S K E L I R N K R E C I R K L K E E I K 420	GAACTGATCATCCCCTGCATTCACTTATCATTAATGACATTTCCCAAGGATGATCTG 3000 E L I I P C I H L I I N N D I S K D D L 1000
ATTCTGCAGCAAAAATTGGAAAGGTATGTGAAATATGGCTCAGGCCCAGCTCGGTTCCCG 1320 I L Q Q K L E R Y V K Y G S G P A R F P 440	GATGCCATTGAGGTCATGAGAAACCATTGGTGCTCTTACCTTGGGCAAGATATTGCAGAA 3060 D A I E V M R N H W C S Y L G Q D I A E 1020
CTCCCGGACATCCTGAAATATGTTATTGAATTTGCTAGTACAAAACCTGCCTCAGAAAGC 1380 L P D M L K Y V I E F A S T K P A S E S 460	AATCTGCAGCTGTGCCTAGGGGAGTTCTACCCAGACTTCTAGATCCTTCTGCAGAAATC 3120 N L Q L C L G E F L P R L L D P S A E I 1040
TGTCCACCTGAAAGTGACACACATATGACATTACCACTTTCTTCAGTGCACTGCTCGGTT 1440 C P P E S D T H M T L P L S S V H C S V 480	ATCGTCTTGAAAGAGCCTCCAACTATTGGACCCAATTCTCCCCTATGACCTATGTAGCCGA 3180 I V L K E P P T I R P N S P Y D L C S R 1060
$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	TTTGCAGCTGTCATGGAGTCAATGGGAGTTCAACTGTGAAGTGAAATAAGCTCCC 3240 F A A V M E S I Q G V S T V T V K * 1077
ACCTTTTCTTCTCCTGAAGATTCTTTACCCAAGTCTAAACCACTGACATCTTCTCGGTCT 1560 T F S S P E D S L P K S K P L T S S R S 520	ACATGTTCAAGGCCCATTCTGGTTCCTGGCTGCCTGCCTCTTGCACAGAAGTTCGTTGTC 3300 ATAGTGCTCACCTTGGCAAAAGGATTAGGTGGGCACATAAGATTCCCGATCAGACCCCCAAC 3360 CATGCTGCCGTCGTGA BACAACATTCAGTGGGCACATTAAGATTCCCGATCAGACCCCCAAC 3360
TCCATGGAAATGCCTTCACAGCCAGGCCAGGCCACGAACAGTCACAGATGAGGAGATAAATTTT 1620 S M E M P S Q P A P R T V T D E E I N F 540	AAAGCTGTTTCACTAGAAAGGCAGAAGCAGTGTTTATAAGGTGTGAATACGCCAGA 3480 AAAAGCTGTATTCACTAGAAAGGCAGAAGCAGTGTTATTAAGGTGTGAATACGGCCAGA 3480 AGACCTGAAATGCCTTGTACCTACAACAATGCTTAGGCTTATTCAAGCCTCTTGCCACTT 3540
GTTAAGACCTGTCTTCAGAGATGGAGGAGGAGGAGATGAACAAGATTAACAAGATTTAAAG 1680 V K T C L Q R W R S E I E Q D I Q D L K 560	TIAMAMITATCUTTCAGGCATAAATATTTTTGACAGCAGAATAGAAGAATGATTCATGAG 3600 AACCTGAACCAGATGAAC 3618



USP28 nucleotide and amino acid sequences (GenBank accession number AF266283). Nucleotides and residues are numbered from the presumptive first ATG (methionine) codon. Specific primers used for cloning are also indicated. The alternative polyadenylation signal is underlined.

11race7 TCGGCC -1

M T A E L Q Q D D A A G A A D G H TGCCAAATGCTGTTAAATCAACTGAGAGAAATCACAGGCATTCAGGACC COMILINOL RETTGTODP CATGAAGCTCTGAAGGCCAGTAATGGTGACATTACTCAGGCAGTCAGCCTT HEALKASNGDITQAVSL

ACTTGTATTGCAAGTACTACTCAGACTATTGAACAGATGTACTGCGATCCTCTCCTTCGT 1740 TCIASTTQTIEQMYCDPLLR580

CAGGTGCCTTATCGCTTGCATGCAGGTTCTTGTTCATGAAGGACAAGCAAATGCTGGACAC 1800

vector-derived forward primers (GR1, GR2). The comparison with the PAC clone PDJ105h16 sequence (htgs database) allowed us to identify the first presumptive methionine. However, as no further in-frame stop codons were detected, the use of another initiation codon could not be ruled out. The search for a putative TATA box in the upstream genomic sequence rendered no positive results.

No cDNA clones with a poly(A)⁺ tail could be isolated, but BlastN comparisons against the dbEST database allowed the identification of an overlapping EST (AI337094) containing the polyadenylation signal.

USP28 cDNA (accession number AF266283) is at least 3,624 bp long and encodes 1,077 amino acids with a relative molecular mass of 122.4 kDa. The gene is organized in 25 exons and exon-intron boundaries were determined after comparison with the human htgs database (Table 1). Average exon length was 130 bp, the shortest being 71 bp and the longest 229 bp. Nucleotide and amino acid identities between USP28 and USP25 (Figure 2) are 55.77% and 51.36%, respectively. USP28 is more similar to USP25 than to any other known UBP. Besides, exon-intron boundaries between USP28 and USP25 are highly conserved (Figure 2). The homology between USP28 and USP25 with other UBP family members appears to be confined to the reported conserved domains.

Expression analysis of USP28 in human tissues

Northern analysis for *USP28* was performed using the cDNA clone 5A11 as probe (Figure 3). A single transcript of approximately 4.5 kb was identified in heart and skeletal muscle after short film exposure. At longer exposures a transcript of the same size was detected in all assayed tissues, albeit at a very low level. To compare the amount of RNA poly(A)⁺ loaded in each lane an actin probe was used as a control.

Alternative splicing of USP25 and USP28

Isoforms generated by alternative splicing have been described for some vertebrate UBPs [13,15]. Sequence analysis of the isolated *USP25* cDNA clones revealed four alternatively spliced exons. Three of those generated an in-frame stop codon in some human fetal brain cDNA clones and were located between exons 3 and 4, 10 and 11, and 17 and 18 (exons numbered according to [14]. The fourth alternatively spliced product corresponded to exon 19 and added 32 amino acids to the protein product (exon 19 has been renamed 19b in this work). It had been previously identified in a single I.M.A.G.E consortium neuroepithelium cDNA clone (id: AA209364 [16]). In order to verify all these putative exons, specific sets of primers (Table 2) were designed for RT-PCR amplification on 24 different tissues (Multiple Tissue cDNA Panels, Clontech).

The additional sequences between exons 3-4 and 17-18 were not further observed in any tissue tested. However, the alternative sequence between exons 10 and 11 (named exon 10b) was clearly detected and verified by sequencing in testis (Figure 4a), where both transcripts (exons 10-11 and 10-10b-11) had been amplified at similar levels. Exon 10b was also detected, albeit faintly, in small intestine, spleen and peripheral leukocytes. This exon encompasses 124 bp and generates a stop codon in the open reading frame (ORF) at nucleotide 1,453. Therefore, this transcript would produce a 361 amino acid truncated protein where the cysteine, but not the histidine UBP domain, would be preserved.

RT-PCR experiments performed with specific primers, 321F-321R (located in exons 17 and 19b, respectively) and N1-121R (in exons 18 and 20, respectively) (Table 2), allowed us to visualize three mRNA isoforms. The shortest, already identified in many expressed sequence tags (ESTs), showed exon 18 directly fused to exon 20 and was detected in all the 24 different tissues tested (Multiple Tissue cDNA Panel, Clontech). The second isoform contained exons 18-19b-20, was of intermediate size and corresponded to the reported USP25 [14]. This isoform was detected in all the assayed tissues except fetal and adult muscle and heart (Figure 4a). Finally, the longest isoform was only detected in fetal and adult muscle and heart mRNAs and included a new exon (named 19a) which was always fused to 19b (18-19a-19b-20) in these tissues (Figure 4a). Exon 19a contained 114 bp that added 38 amino acids to the reported USP25 protein.

Primers 5A11.2 and 5A11.1 from USP28 (Table 2), located respectively in exons 16 and 24, were used to verify the homolog of the USP25 muscle and heart isoform (exons 18-19a-19b-20). RT-PCRs were performed in different tissues and the amplified products were subcloned and sequenced. Two USP28 sequences were detected in adult and fetal muscle, heart and brain. The shortest fragment corresponded to the reported USP28 cDNA containing exon 19b (Figure 4b). The longest contained a new exon (named 19a) (Figure 4b), which introduced 62 amino acids to the reported ORF. The tissue-specific exon 19a from USP25 (38 amino acids) and USP28 (62 amino acids) showed 41.6% amino acid identity. In contrast to USP25 exon 19b, the exon 19b of USP28 (sharing 53.1% amino acid identity) was not alternatively spliced, as it was always present in the transcripts from all tissues assayed.

Assay for ubiquitin-specific protease activity

To test the ubiquitin-specific protease activity of USP28, USP25 and the muscle and heart USP25 isoform (iUSP25), these enzymes were synthesized as inducible fusion proteins in the pGEX-4T-1 vector (pGEX-USP25; pGEX-iUSP25 and pGEX-USP28). *Escherichia coli* XL1blue cells were cotransformed with each construct and a plasmid expressing the fusion protein Ub-M- β -gal, a synthetic substrate for UBP. Immunoblot analysis using anti- β -galactosidase (β -gal) antibodies showed an efficient cleavage of ubiquitin from the Ub-M- β -gal fusion protein (Figure 5, lanes 5, 6 and 7). As expected, cells with the control pGEX-4T-1 vector failed to

Table I

Genomic organization of USP28

	0			
	Exon	Acceptor splice site	Donor splice site	Intron length (bp)
I	57		GCTCG/ gt gggctccg	>14,000
2	58-135	aaatatag ag /AGCTA	TGAAG/ gt tggtttcc	1,635
3	136-268	tctcccat ag /GCCAG	AGCAA/ gt aggtacca	10,737
4	269-374	tatcttac ag /AAGTT	AACAG/ gt tattgatt	905
5	375-534	cacaatat ag /GATGC	TTCAG/ gt atgatatt	6,262
6	535-621	tttatttc ag /TCTCT	ATACA/ gt aagttggt	691
7	622-759	ttttctac ag /GAAAA	AGCAG/ gt acaaaaga	1,426
8	760-833	tctcttat ag /CAAGA	GTTAA/ gt gagtgttg	976
9	834-910	ctaaaagc ag /CAGTC	TGAAG/ gt aaattctc	1,515
10	911-1,059	ctttgcct ag /GAAAA	AAGAG/ gt acgttgga	1,836
11	1,060-1,187	ttgacact ag /CGTTG	GACAG/ gt gagttctt	3,532
12	1,188-1,283	atttactt ag /GTACA	GAAAG/ gt gagttttg	5,763
13	1,284-1,463	tgtcgctt ag /GTATG	GAAAG/ gt aatgaaaa	2,282
14	1,464-1,672	tcagtaca ag /TACAA	ACAAG/ gt tggctctt	1,213
15	1,673-1,743	ttccaaac ag /ATTTA	GTCAG/ gt agaatgaa	1,380
16	1,744-1,972	tgacttac ag /GTGCC	TGCAG/ gt aaaagtat	3,021
17	1,973-2,164	ttttcctc ag /AGGCA	ACAAG/ gt atctgaga	625
18	2,165-2,304	tctttatc ag /AGCCT	GTGAG/ gt gagaatga	1,713
19	2,305-2,400	cttgtggt ag /GTGAT	TTAAG/ gt atctctgt	1,443
20	2,401-2,579	cccttaat ag /GCATT	GAAAG/ gt gagaagag	116
21	2,580-2658	tcccacac ag /ATCAA	ACAAG/ gt aaagtgtt	795
22	2659-2,738	tctttttc ag /AAGTG	GGAAA/ gt atgttggc	516
23	2,739-2,862	tttttcta ag /GTACC	TTCTG/ gt ttgtactg	1,479
24	2,863-3,058	ttatctgt ag /GAGCT	TGCAG/ gt atatgctc	2,068
25	3,059	ccctccac ag /AAAAT		

The donor and acceptor splice site signals are indicated in bold.

cleave Ub-M- β -gal (lane 4). The endogenous *E. coli* XL1-blue β -galactosidase (lacking the α peptide, and thus producing a shorter protein) was also detected (lanes 2 and 8).

Overexpression of USP25 in Down syndrome patients

As *USP25* is located on chromosome 21, its overexpression could be presumed in Down syndrome (DS) patients. In order to assess the expression level of *USP25* in DS versus control samples, we analyzed eight independent fetal brain samples (four trisomic and four disomic) by real-time quantitative PCR. An average of 1.7-fold overexpression of *USP25* was shown in trisomic versus disomic samples.

Discussion

Database homology searches with the reported *USP25* [14] have led to the characterization of a new UBP member in the human genome, named *USP28*, which maps at 11q23. Structural comparisons at the genomic and protein levels of

*USP*25 and *USP*28, and the deubiquitinating enzymatic assays, allowed the definition of a new UBP subfamily.

Sequence alignments of UBPs have been hindered by the few shared conserved segments, which need specific computer programs to be identified. Nonetheless, several UBP subfamilies have been reported so far. DUB1 and DUB2, with 88.4% amino acid identities, is one of those. Similarly, alignments of UBP41, UBP46, UBP52 and UBP66 from chick skeletal muscle suggest a new subfamily. In our case, the alignment of the newly reported USP28 with USP25 showed homologies beyond the conserved UBP domains and amino acid identities amounting to 51%. In addition, the extensive alignment at the amino-terminal segment supports the proposed USP28 translation initiation site. USP25 and USP28 share the exon-intron distribution and those intron positions with an inaccurate match, located between exons 1-2, 13-14 and 17-18 (Figure 2), could be explained by slippage of the donor or acceptor splice sites. All these data strongly

USP28	WTAELQQDDAAGAADGHGSSCOMLLNQLREITGIQDPSFLHEALKASNGDITQAVSLLTD	60
USP25	MTVE.QNVLQQSAAQKHQQTFLNQLREITGINDTQILQQALKDSNGNLELAVAFLTA	56
USP28	ERVKEPSQDTVATEPSEVEGSAANKEVLAKVIDLTHDNKDDLQAAIALSLLES	113
USP25	KNAKTEQQEETTYYQTALPGNDRYISVGSQADTNVIDLTGDDKDDLQRAIALSLAESNRA	116
USP28	PKIQADGRDLNRMHEATSAETKR.SKRKRCEVWGENPNPNDWRRVDGWPVGLKNVGN	169
USP25	FRETGITDEEQAISRVLEASIAENKACLKRTPTEVWRDSRNPYDRKRQDKAPVGLKNVGN	176
USP28	TCWFSAVIQSLFQLPEFRRLVLSYSLPQNVLENCRSHTEKRNIMFMQELQYLFALMMGSN	229
USP25	TCWFSAVIQSLFNLLEFRRLVLNYKPPSNAQDLPRNQKEHRNLPFMRELRYLFALLVGTK	236
USP28	RKFVDPSAALDLLKGAFRSSEEQQQDVSEFTHKLLDWLEDAFQLAVNVNSPRNKSENPMV	289
USP25	RKYVDPSRAVEILKDAFKSNDSQQDVSEFTHKLLDWLEDAFQMKAEEETDEEKPKNPMV	296
USP28	QLFYGTFLTEGVREGKPFCNNETFGQYPLQVNGYRNLDECLEGAMVEGDVELLPSDHSVK	349
USP25	ELFYGRFLAVGVLEGKKFENTEMFGQYPLQVNGFKDLHECLEAAMIEGIESLHSENSGK	356
USP28	YGQERWFTKLPPVLTFELSRFEFNQSLGQPEKIHNKLEFPQIIYMDRYMYRSKELIRNKR	409
USP25	SGQEHWFTELPPVLTFELSRFEFNQALGRPEKIHNKLEFPQVLYLDRYMHRNREITRIKR	416
USP28	ECIRKLKEEIKILQQKLERYVKYGSGPARFPLPDMLKYVIEFASTKPASESCP.PESDTH	468
USP25	EEIKRLKDYLTVLQQRLERYLSYGSGPKRFPLVDVLQYALEFASSKPVCTSPVDDID	473
USP28	MTLPLS.SVHCSVSDQTSKE.STSTESSSQDVESTFSSPEDSLPKSKPLTSSRSSMEMP	525
USP25	ASSPPSGSIPSQTLPSTTEQQGALSSELPSTSPSSVAAISSRSV.IHKPFTQSRIPPDLP	532
USP28	SQPAPRTVTDEEINFVKTCLQRWRSEIEQDIQDLKTCHASTTQTIEQMYCDPLLRQVPYR	585
USP25	MHPAPRHITEEKLSVLESCLHRWRTEIENDTRDLQESISRIHRTIELMYSDKSMIQVPYR	592
USP28	LHAVLVHEGQANAGHYWAYIYNQFRQS.WLKYNDISVTESSWEEVERDSYGGLRNVSAYC	644
USP25	LHAVLVHEGQANAGHYWAYIFD.HRESRWMKYNDIAVTKSSWEELVRDSFGGYRNASAYC	651
USP28	LMYINDKLPWFNAEAAPTESDO.MSEVEALSVELKHYIQEDNWRFEQEVEEWEEEQSCKI	703
USP25	LMYINDKAQFLIQEFNKETGOPLVGIETLPPDLRDFVEEDNQRFEKELEEWDAQLAQKA	711
USP28	POMESSTNSSSQDYSTSO PSVASSHGVRCLSSEHAVIVKEOTAOAHANTARA	756
USP25	LQEKLLASQKLRESETSVITAOAAGDEYLEQP.SRSDFSKHKEETIQIITKASHE	767
USP28	YEKSGVEAALSEVMLSPAMQGVILAIAKARQTFDRDGSEAGLIKAFHEEYSRLYQLAKET	816
USP25	HEDKSPETVLQSIMMTPNMQGIIMAIGKSRSVYDRCGPEAGFFKAIKLEYARLVKLAQED	827
USP28	PTSHSDPRLQHVLVYFFQNEAPKRVVERTLLEQFADKNLSYDERSISIMKVAQAKLKEIG	876
USP25	TPPETDYRLHHVVVYFIQNQAPKKIIEKTLLEQFGDRNLSFDERCHNIMKVAQAKLEMIK	887
USP28	PDDMNMEEYKKWHEDYSLFRKVSVYLLTGLELYQKGKYQEALSYLVYAYQSNAALLMKGP	936
USP25	PEEVNLEEYEEWHQDYRKFRETTMYLIIGLENFQRESYIDSLLFLICAYQNNKELLSKGL	947
USP28	RRGVKESVIALYRRKCLLELNAKAASLFETNDDHSVTEGINVMNELIIPCIHLIINNDIS	996
USP25	YRGHDEELISHYRRECLLKLNEQAAELFESGEDREVNNGLIIMNEFIVPFLPLLLVDEME	1007
USP28	KDDLDAIEVMRNHWCSYLGQDIAENLQLCLGEFLPRLLDPSAEIIVLKEPPTIRPNSPYD	1056
USP25	EKDILAVEDMRNRWCSYLGQEMEPHLQEKLTDFLPKLLDCSMEIKSFHEPPKLPSYSTHE	1067
USP28 USP25	LCSRFAAVMESIQGVSTVTVK 1077 LCERFARIMLSLS.RTPADGR 1087	

Figure 2 Protein alignment of USP25 and USP28. Amino acid identities are boxed in black and conserved amino acid changes are boxed in gray. The exon-intron boundaries in each gene are marked by arrowheads.



Figure 3

Northern analysis of USP28 in adult human tissues. Molecular weight marker sizes are indicated. Hybridization with an actin probe used as control is shown below.

support a common ancestry for *USP25* and *USP28*, and suggest that the minor variations observed may contribute to functional differences.

Northern analysis showed abundant *USP28* expression in adult skeletal muscle and heart (Figure 3). The transcript size (4.5 kb approximately) was longer than expected from

Table 2

Primers used in the splicing analysis of USP25 and USP28					
Primer name	Sequence 5'-3'	Position in cDNA (nucleotides)			
261F	GCCATGACCGTGGAGCAGAACG	367-388			
261R	CAGCACTAAACCAACAAGTATTGCCA	891-916			
1.2F	GAAGCCAGCATAGCAGAGAATAAAGC	769-794			
1.2R	CACAGGTGGTAATTCAGTAAACCAATG	1,450-1,476			
321F	GGGATGCACAACTTGCCCAG	2,478-2,496			
321R	CCCTGCTTCAGGGCCACACCTG	2,772-2,793			
NI	AGGAGACCCAGAATAT	2,574-2,589			
121R	CAACCTTGCATATTCCAACT	2,810-2,829			
*5A11.1	GCTGCTTTGGCATTCAGC	1,947-1,968			
*5A11.2	TGACAAACTACCCTACTTCAATG	2,804-2,882			

*These primers were used in the splicing analysis of USP28.

cDNA analysis, although this variability could be due to distinct polyadenylation and transcription initiation sites, as reported for *USP25* and other UBPs [17].

The high specificity attributed to individual members of the ubiquitin proteolytic system and the structural complexity of USP25 and USP28 prompted us to analyze the tissue-specific mRNA isoforms and the cellular localization of the proteins. Alternative splicing had been also reported for other UBP members, such as USP₃ [17], USP₄ (previously named UnpEL-UnpES [13]), USP5 (previously named ISOT-1/2 [18-20]), USP9X (previously named DFFRX [15]) and USP15 [9]. Some cDNA clones of USP25 and USP28 contained additional exons that introduced in-frame stop codons, similarly to what has been reported for USP15 and USP3. These transcripts most probably originated from splicing errors and were fortuitously cloned, as they did not appear after northern or RT-PCR analyses. Besides, the absence of the conserved domains in the truncated proteins would compromise functionality. Nonetheless, the USP25 RT-PCR assays in testis produced comparable amplification levels of sequences containing either exons 10-10b-11 (10b introduces an in-frame stop codon) or exons 10-11. This argues in favor of a tissue-specific function for the truncated protein, possibly related to substrate availability and/or enzyme activity.

Exon 19b is present in all USP_{28} transcripts in all the assayed tissues. In constrast, exon 19b from USP_{25} is present in all tissues but not in all transcripts, thus constituting an alternatively spliced exon. The high degree of sequence homology of exon 19b from both genes supports its functional relevance. In addition, the tissue specificity shown for exon 19a of USP_{25} (muscle and heart) and USP_{28} (muscle, heart and brain) could confer the enzyme a tissue specificity to deubiquitinate a ubiquitous substrate. This would apply to a widely expressed gene whose function was only relevant in some tissues, as suggested for Fam [20]. Alternatively, the tissue-specific exon would bestow on the enzyme the ability to recognize a tissue-specific substrate, as suggested for Faf [20].

The specific subcellular localization reported for some deubiquitinating enzymes may imply spatial restriction of either the locus of action or the accessibility to the substrate but it might also indicate regulation of cellular processes where ubiquitylation plays a role unrelated to protein degradation (for a recent review see [21]). In our case, preliminary subcellular localization experiments with protein fusions to GFP showed that USP25 was cytosolic (data not shown) and did not support an involvement outside the ubiquitin-proteasome pathway.

Although several deubiquitinating enzymes have been shown to contribute to development and differentiation (that is, Faf (*Drosophila* Fat facets) [15] and UBP43 [22]), the specific function of most family members remains unknown. Homology searches of the domains conserved



Figure 4

Alternatively spliced exon analysis of USP25 and USP28. (a) The specific USP25 isoforms of testis (exons 10a-10b-11) and heart and muscle (18-19a-19b-20). (b) The specific USP28 isoform of heart, muscle and brain (18-19a-19b-20).

between different UBPs would help to elucidate the function of new members and define the substrate-specific domains. The contribution of *USP25* to Down syndrome pathogenesis is still unclear. However, its overexpression (1.7-fold \pm 0.13, P < 0.05 according to the Mann-Whitney test) in Down syndrome with respect to control fetal brain samples would support its involvement in the pathology. In fact, several UBPs have shown gene-dosage effects, such as USP9Y (whose gene is located in the Y-chromosome pseudoautosomal region and is involved in male azoospermia [23]) and other USPs related to an euploid syndromes: DFFRX in Turner syndrome [15] and USP18 in DiGeorge syndrome [24]. On the other hand, *in vitro* overexpression or inhibition of some ubiquitin-specific proteases has led to programmed cell death, supporting the idea that their activity is dose dependent [25].

Materials and methods USP28 cDNA cloning

To screen cDNA libraries from human fetal brain, placenta and kidney (Clontech), specific primers of the USP-like region



Figure 5

Deubiquitinating activity assay for USP25, the muscular USP25 isoform and USP28. The immunoblot was detected using anti- β -galactosidase (β -gal) antibodies. The sizes corresponding to Ub- β -gal, β -gal and endogenous deleted β -gal are shown by black arrows. Arrowheads on the lanes show the size of the Ub- β -gal fusion protein and the deubiquitinated β -gal. Lane 1, molecular weight marker containing wild-type β -gal; lane 2, untransformed XL1blue (negative control); lanes 3 and 4, XL1blue cells transformed, respectively, with pAC-M- β -gal alone or pAC-M- β -gal and empty pGEX vector; lanes 5, 6 and 7, XL1blue cells transformed, respectively, with pAC-M- β -gal and USP25, pAC-M- β -gal and muscular USP25 isoform, and pAC-M- β -gal and USP28; lane 8, XL1blue cells transformed with the empty pGEX vector.

on chromosome 11 were designed (Figure 1) after comparison of *USP25* cDNA against AC002036, a PAC that contained genomic DNA from chromosome 11. Approximately, 10⁶ phages of each library were plated and transferred onto Nylon membranes (HybondN, Amersham-Pharmacia Biotech).

Probes were labeled by random hexamer priming with [α -³²P]dCTP. A solution containing 50% (v/v) formamide, 5x SSC, 5x Denhart's, 0.1% (w/v) SDS and 100 µg/ml denatured salmon sperm was used to pre-hybridize and hybridize the filters for 2 h and 18 h, respectively. Washes were performed as follows: two washes in 2x SSC/0.1%SDS at 65°C for 10 min and two times in 1x SSC/0.1%SDS at 65°C for 10 min. Filters were exposed during two days with double screen in order to amplify the positive signals. Positive clones were subcloned into pBLUESCRIPT SK+ (Stratagene), sequenced in an ABI₃₇₇ automatic sequencer (Applied Biosystems) and analyzed at the NCBI BLAST server [26]. As only truncated cDNA clones were initially isolated, additional screenings were needed to identify the full-length cDNA.

Northern blot analysis of USP28

The cDNA clone 5A11 (from nucleotides 1,473 to 3,618, Figure 1) was used to probe a human multiple tissue northern blot (Clontech). Hybridization and washes were carried out according to the manufacturer's protocol. A control actin probe was used to assess the amount of RNA poly(A)⁺ loaded in each lane.

Specificity of alternative splicing events

A set of primers corresponding to exon sequences of *USP25* and *USP28* (Table 2) (USP25 primers numbered according to [14]) were designed for RT-PCR experiments on 24

samples from different tissues (Multiple Tissue cDNA Panels, Clontech). Amplification was performed in a total volume of 25 μ l containing: 2.5 μ l of template cDNA, 200 μ M dNTPs, 5 pmol of each primer, 1x Taq Platinum buffer, 1.5 mM MgCl₂ and 1U Taq Platinum (GIBCO-BRL). After denaturing at 94°C for 3 min, two-step PCR was carried out for 35 cycles at 94°C for 30 sec and 58°C for 30 sec. Final extension was for 5 min at 72°C. Bands differing from the expected size were subcloned in pBLUESCRIPT SK+ (Stratagene) and subsequently sequenced in an ABI377 automatic sequencer (Applied Biosystems). Sequences were analyzed using the BLAST software at the NCBI [26,27].

Assay for ubiquitin-specific protease activity

The ubiquitin-specific protease activity of USP25, USP25specific heart and muscle isoform, which contains the additional exon 19a (USP25 isoform), and USP28 was determined as described [10,11]. The three corresponding cDNAs were cloned in-frame in pGEX-4T-1 (Amersham Pharmacia Biotech) downstream from the glutathione-*S*-transferase (GST) coding region. Plasmid pACY184 Cm^r expressing Ub-Met- β -gal protein fusion (pAC-M- β -gal) was kindly provided by M. Hoschtrasser. *E. coli* XL1blue bacteria were co-transformed with pAC-M- β -gal and either pGEX-4T-1-USP25 or pGEX-4T-1-USP25 isoform or pGEX-4T-1-USP28. Amp^r and Cm^r colonies were grown and induced for 3 h with isopropyl- β -thiogalactopyranoside (final concentration 1 mM). Total protein extracts were analyzed by western blotting with anti- β -galactosidase rabbit polyclonal antibody (Cappel).

Overexpression of USP25 in Down syndrome patients

Total RNA from 5 mg of fetal brains of Down and control samples (three samples each) were obtained using ABI PRISM 6700 Automated Nucleic Acid workstation. RT-PCRs were produced according to Tagman Reverse transcription reagents (Roche Molecular Systems). Quantitative PCRs were performed using the Universal Master Mix (Applied Biosystems) following the manufacturer's specifications. The real-time amplification was analyzed by the ABI Prism 7700 Sequence detection system. USP25 primers and the Taqman probe (using FAM as reporter and TAMRA as quencher) were designed according to the Primer Express software. Forward 5'-GATGAAAGGTGTCACAACATAATGAAA-3'; primer: reverse primer: 5'-CCACTCCTCATATTCCTCCAAGTTT-3'; TaqMan probe: 5'-TCAAGCCAAACTGGAAATGATAAAACCT-GAAGA-3'. To normalize the USP25 quantitative determinations, GAPDH (Applied Biosystems) was used as endogenous control. The standard deviation of the disomic and trisomic samples was 0.08 and 0.13, respectively. The overexpression (1.7 fold) of USP25 in trisomic fetal brains shows statistical significance according to the Mann-Whitney test (P < 0.05).

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