Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 314 (2004) 54–62
BBRC
www.elsevier.com/locate/ybbrc

Cloning and enzymatic analysis of 22 novel human ubiquitin-specific proteases☆,☆☆

Víctor Quesada,[1] Araceli Díaz-Perales,[1] Ana Gutiérrez-Fernández, Cecilia Garabaya, Santiago Cal, and Carlos López-Otín*

Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Instituto Universitario de Oncología, Universidad de Oviedo, 33006-Oviedo, Spain

Received 3 December 2003

Abstract

We have identified and cloned 22 human cDNAs encoding novel members of the ubiquitin-specific protease (USP) family. Eighteen of the identified proteins contain all structural features characteristic of these cysteine proteinases, whereas four of them have been classified as non-peptidase homologues. Northern blot analysis demonstrated that the identified USPs are broadly and differentially distributed in human tissues, some of them being especially abundant in skeletal muscle or testis. Enzymatic studies performed with the identified USPs revealed that at least twelve of them are deubiquitylating enzymes based on their ability to cleave ubiquitin from a ubiquitin-β-galactosidase fusion protein. These results provide additional evidence of the extreme complexity and diversity of the USP proteolytic system in human tissues and open the possibility to explore the relevance of their multiple components in the regulation of ubiquitin-mediated pathways in normal and pathological functions.

Keywords: Cancer; Degradome; Protease; Proteasome; Ubiquitin; USP

The modification of cellular proteins by ubiquitin and ubiquitin-like proteins is an essential regulatory mechanism in many biological processes such as cell cycle progression, DNA-repair, organelle biogenesis, vesicular trafficking, transcriptional activation, signal transduction, and intracellular proteolysis [1–3]. This post-translational modification is a dynamic and reversible process controlled by the coordinate action of multiple ubiquitin-conjugating and deubiquitylating enzymes. The conjugation of ubiquitin and related molecules to proteins is catalyzed by the successive actions of three types of enzymes: ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin-protein ligase (E3) enzymes. The conjugation of all ubiquitin-like proteins—such as SUMO/Sentrin/Smt3p, UCRP/ISG15, NEDD8/Rub1 and Apg12—to their respective substrates also proceeds through this three-step cascade mechanism involving E1, E2, and E3 enzymes [4,5]. The removal of the ubiquitin and ubiquitin-like domains is catalyzed by processing proteases that have been generically named deubiquitylating enzymes (DUBs). There are at least seven protease families with ability to participate in the deconjugation of ubiquitin and ubiquitin-like adducts: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), sumo-specific proteases (SENPs), autophagins, otubains, ataxin-3/josephin ubiquitin proteases, and JAMM isopeptidases [6–13]. Among this growing group of structurally diverse proteases, the USPs have raised special interest due to the multiple family members described in different eukaryotic organisms and to the different functions ascribed to these enzymes in both normal and pathological conditions.

☆ *Abbreviations:* bp, base pair(s); PCR, polymerase chain reaction; USP, ubiquitin-specific protease.

☆☆ This work was supported by grants from Comisión Interministerial de Ciencia y Tecnología-Spain, Gobierno del Principado de Asturias-Spain, Fundación "La Caixa" and European Union; V.Q. is the recipient of a fellowship from Ministerio de Ciencia y Tecnología, Spain. The Instituto Universitario de Oncología is supported by Obra Social Cajastur-Asturias.

* Corresponding author. Fax: +34-985-103564.
E-mail address: clo@correo.uniovi.es (C. López-Otín).

[1] Both these authors have contributed equally to this manuscript.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.12.050

USPs are cysteine proteases that vary greatly in size and structural complexity [6,14–16]. They contain several conserved regions in their amino acid sequence, including those surrounding the Cys, His, and Asp/Asn residues that form part of the catalytic triad of these enzymes. Outside of the core catalytic domain, USPs exhibit N-terminal and C-terminal extensions that have been proposed to play roles in determining cellular localization and substrate specificity of these enzymes. USPs may act at multiple levels in the ubiquitin pathway [6,14–16]. Thus, these proteases are responsible for the generation of free ubiquitin from precursor fusion proteins. USPs also have the ability to remove ubiquitin from a ubiquitylated substrate, thereby regulating the localization and activity of this substrate. Likewise, these cysteine proteases remove polyubiquitin from target proteins, rescuing them from degradation by the proteasome. Finally, USPs may also participate in the editing of polyubiquitin chains or remove polyubiquitin from proteasome degradation products and recycle ubiquitin from these disassembled chains by hydrolyzing the isopeptide-linked ubiquitin units [6,14–16].

Consistent with the multiple levels of actuation of USPs, these enzymes have been linked to a variety of biological and pathological processes such as regulation of cellular growth pathways [17–21], stabilization of the p53 tumor suppressor [22], inhibition of chromatin-mediated gene silencing [23]; or modulation of developmental processes and thyroid hormone activation [24–28]. The finding that USPs may be involved in such diverse processes has stimulated the search for new family members that could play additional yet uncharacterized functions. Recently, in the course of studies aimed at the annotation of the human, mouse, and rat degradomes—the complete repertoire of proteases of these organisms–, we observed the presence of a number of DNA regions with ability to code for putative novel USPs [29,30]. In this work, we report the identification and structural characterization of 22 new human USPs. We also analyze the tissue distribution of these novel USPs and perform a preliminary analysis of their enzymatic activity.

Materials and methods

Materials. Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals (Mannheim, Germany). Double-stranded DNA probes were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) from Amersham–Pharmacia Biotech (Buckinghamshire, UK), using a commercial random-priming kit purchased from the same company. cDNA libraries and filters containing polyadenylated RNAs from human tissues were from Clontech (Palo Alto, CA).

Database screening and cDNA cloning. The advanced BLAST program from the NCBI was used to search human genome databases, looking for regions encoding putative proteins with sequence similarity to previously described members of the USP family of proteases. This computer search led us to identify several DNA contigs containing regions with significant sequence similarity to USPs. To obtain cDNA sequences corresponding to the putative proteins encoded by these DNA contigs, we designed specific oligonucleotides for each of them and performed PCR experiments using a panel of commercially available cDNA libraries from lung, kidney, endothelium, and brain (Clontech) and the Expand High Fidelity PCR system (Roche Molecular Biochemicals). The obtained cDNAs were cloned into pBluescript vector and characterized by nucleotide sequencing, using an ABI-Prism 310 DNA sequencer (Applied Biosystems).

Northern blot analysis. Nylon filters containing 2 μg of poly(A)$^+$ RNA of a wide variety of human tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5× SSPE, 10× Denhardt's solution, 2% SDS, and 100 μg/ml of denatured herring sperm DNA, and then hybridized with radiolabeled probes for each cloned cDNA. Hybridization was performed for 20 h under the same conditions. Filters were washed with 0.1× SSC, 0.1% SDS for 2 h at 50 °C and exposed to autoradiography.

Enzymatic assays for deubiquitylating activity. Enzymatic activity of recombinant USPs was tested as previously described by Baker et al. [31]. Briefly, isolated cDNAs for USPs were cloned in the expression vector pGEX-3X (Amersham Biosciences). The resulting vectors were cotransformed with pACY184 (provided by Dr. M. Hochstrasser, Yale University) in *Escherichia coli* XL1-Blue competent cells. pACY184 produces a ubiquitin-β-galactosidase fusion protein, which can act as a substrate for USPs. Expression of fusion proteins was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (final concentration 1 mM) for 4 h at 28 °C. Total protein extracts were analyzed by Western blotting with anti-β-galactosidase mouse monoclonal antibody (Promega).

Results

Identification and cloning of cDNAs encoding 22 novel human USPs

To identify novel members of the USP family of cysteine proteinases, we used the BLAST algorithm to screen the human genome databases looking for DNA sequences encoding putative proteins similar to previously described family members. This search allowed us to identify a series of DNA contigs in chromosomes 1p, 2p, 2q, 4q, 6p, 6q, 7p, 10q, 11p, 11q, 12q, 15q, 16p, 17p, 17q, 22q, and Xp, that contained coding information for yet uncharacterized USPs. To generate cDNA clones for each of them, we carried out PCR amplifications using a panel of human cDNA libraries and specific oligonucleotides derived from the identified genomic sequences. Conceptual translation confirmed that these cDNAs coded for novel USPs (Fig. 1 and EMBL Accession Nos. AJ586136, AJ586135, AJ586138, AJ586137, NP 079366, NP 065986, NP 115946, AJ583821, AJ586979, AJ583817, NP 115523, AJ583819, NP 073743, NP 060414, AJ586139, AJ583818, AJ583823, NP 055686, AJ583824, and AJ583820). Several cDNA sequences closely related or identical to those here identified for human USPs, and derived in most cases of large-scale sequencing projects, have also been deposited in publicly available databases (i.e., Accession Nos. XP 353552 and AAP30832).

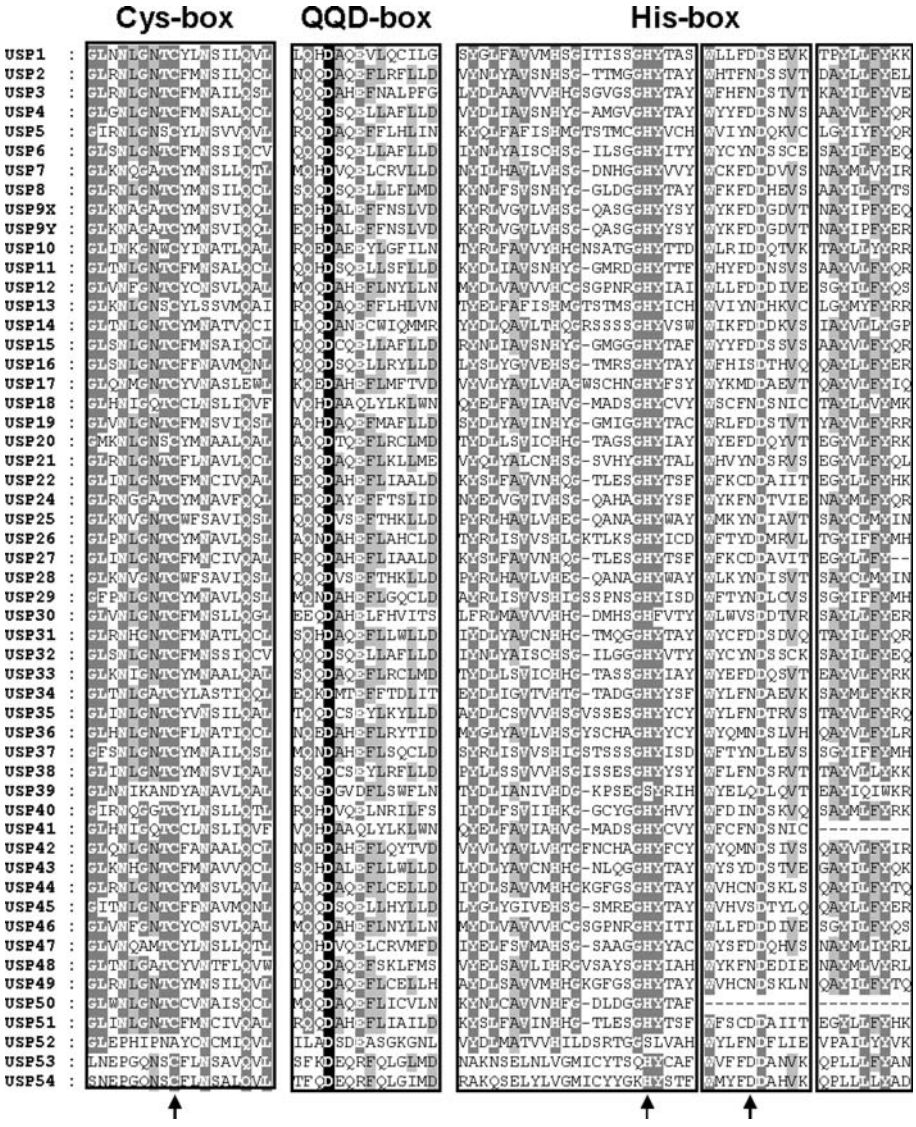

Fig. 1. Amino acid sequence of human USPs around regions important for catalytic activity. The multiple alignment was performed with the CLUSTALX program and edited with Genedoc using information from the local alignment tool MEME (http://meme.sdsc.edu/meme/website/meme.html). Gaps are indicated by *hyphens*. Common residues to all sequences are *shadowed*. The Cys, His, and Asp/Asn residues characteristic of these cysteine proteinases are indicated with an arrow.

Most of these proteins have also been annotated in the protease database MEROPS (http://www.merops.ac.uk) and in our own database of human, mouse, and rat proteases (http://web.uniovi.es/degradome) as members of the C19 family of cysteine proteases. However, no report describing the cloning and characterization of any of these human cDNAs or of their encoded USPs has been published yet. The officially approved names for these enzymes by the Human Genome Organization Nomenclature Committee are: USP30, −31, −34, −35, −36, −37, −38, −40, −41, −42, −43, −44, −45, −46, −47, −48, −49, −50, −51, −52, −53 and −54. USP32, −33, and −39 have been recently described by Paulding et al., Li et al., and Lygerou et al., respectively [26,32,33].

An amino acid sequence alignment of the catalytic domains of the identified human USPs confirmed that they maintain a significant sequence similarity with the equivalent regions of previously characterized family members (Fig. 1). Thus, these USPs exhibit a number of specific structural features characteristic of this family of cysteine proteases including the Cys box, that contains a conserved cysteine residue essential for the catalytic properties of these enzymes [6,14–16]. Interestingly, this cysteine residue is replaced by alanine in USP52 (Fig. 1). Owing to this substitution, this USP is unlikely to have any significant proteolytic activity and it has been classified as a non-protease homologue according to the MEROPS database nomenclature. This Cys box characteristic of USPs is followed by a second region of

similarity named the "QQD" box [34], which contains an aspartic acid residue absolutely conserved in all USPs identified herein. The third region of significant sequence similarity is called the His box and contains a histidine that has also been proposed to participate in the catalytic mechanism of USPs [6,14–16]. This His residue is absolutely conserved in all USPs described in this work with the exception of USP52, thereby reinforcing its classification as a non-protease homologue of the USP family. Several residues C-terminal to this His residue, there is an Asp/Asn residue which has been proposed to act as the third component of the catalytic triad of USPs [35]. This Asp/Asn residue is conserved in all USPs identified herein, with the exceptions of USP30 and USP45 which contain a Ser residue at this position as previously described for the catalytically active USP16 [21], and USP50 which lacks the entire C-terminal region encompassing this third member of the catalytic triad (Fig. 1). On this basis, USP50 has also been classified as a non-protease homologue of USPs. Likewise, USP53 and USP54 despite having all three residues of the catalytic triad lack an essential His residue located nine amino acids N-terminal to the catalytic His of USPs [35] and show a marked divergence in other conserved regions of the catalytic domain. Accordingly, both USP53 and USP54 have also been included in the category of non-protease homologues of the USP family. Finally, the detailed sequence alignment between human USPs also revealed that the amino acid sequences surrounding these conserved Cys, Asp, and His residues are highly conserved between all of these enzymes, and include a Gln residue located very close to the catalytic Cys, which can be part of the oxyanion hole present in the structure of cysteine proteases, including deubiquitylating enzymes [35–37] (Fig. 1).

In marked contrast with this overall sequence similarity in the catalytic domains of the identified USPs, and as previously reported for other family members, there are notable divergences in both the N- and C-terminal ends of these proteins. This fact may be explained at least in part by the presence of a series of recognizable ancillary domains in the corresponding N- and C-terminal regions of the identified USPs. As shown in Fig. 2, the Zn-finger ZnF-UBP domain is present in four of the identified USPs (USP44, −45, −49, and −51); the DUSP (domain in ubiquitin-specific proteases) and UBQ (ubiquitin-like) domains are found in USP48; the ubiquitin-interacting motif (UIM) in USP37; and the exonuclease III (ExoIII) domain in USP52. It is also remarkable the presence in some of these USPs of archetypal sorting signals that may direct these enzymes to specific intracellular locations.

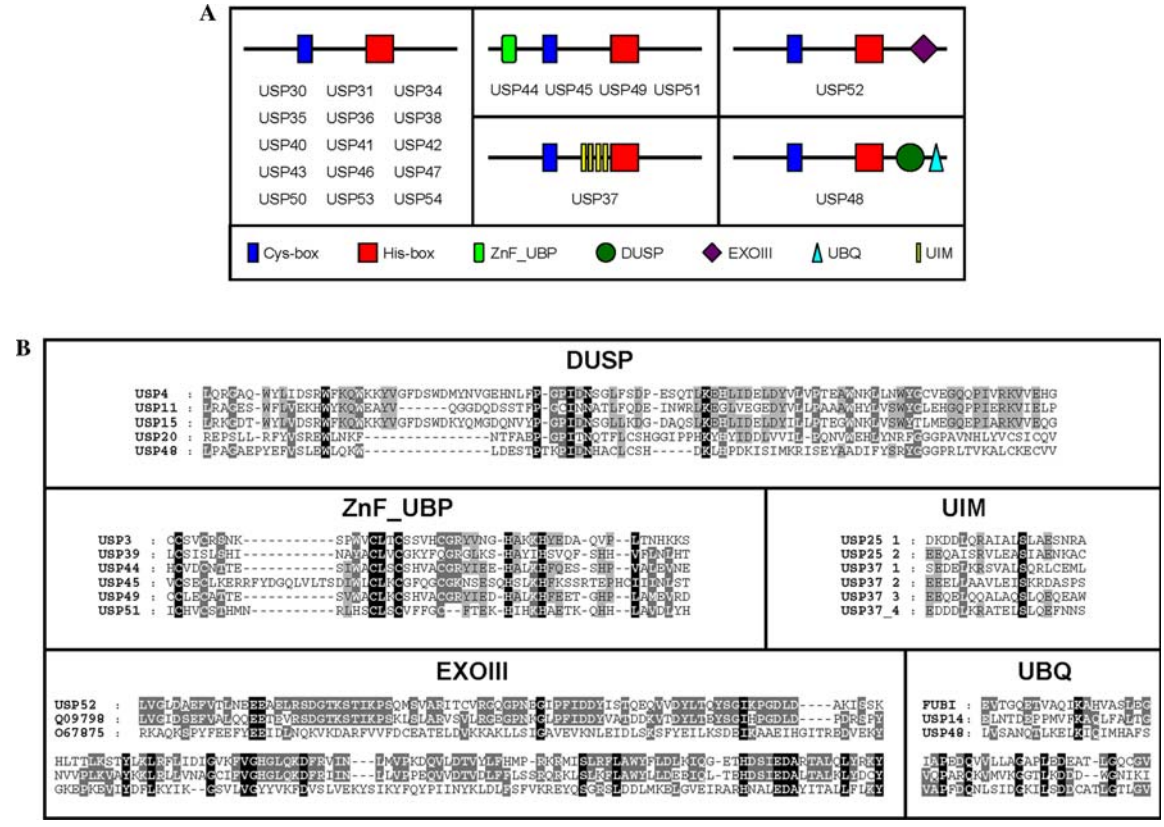

Fig. 2. Domain organization of the identified human USPs. (A) Schematic representation of the domains present in the identified USPs. (B) Amino acid sequence alignment of the protein domains shown in (A).

Thus, several of them present nuclear localization signals, whereas USP30 shows a mitochondrial targeting sequence.

Expression analysis of the identified USPs in human tissues

As a preliminary step to study the physiological role of USPs in human tissues, we examined by Northern blot analysis the expression pattern of these genes in a variety of tissues including colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart. The filters containing poly(A)$^+$ RNA from these tissues were sequentially hybridized with radiolabeled cDNA probes for the identified USPs and the results obtained are shown in Fig. 3. USP36, −40, −42, −46, −48, and −52 are widely distributed in human tissues. By contrast, USP30, −31, −35, −38, −44, −45, −47, −50, −53, and −54 are only produced at significant levels by one or a few tissues. Finally, transcripts for USP34, −37, −41, −43, −49, and −51 are not detected by Northern blot analysis, although they can be amplified by RT-PCR from different human tissues: USP34 from brain; USP37 from brain and prostate; USP43 from brain, aorta, and lung; USP49 from samples derived of tumor biopsies; and USP41 and USP51 from prostate, brain, lung, aorta, and kidney (data not shown).

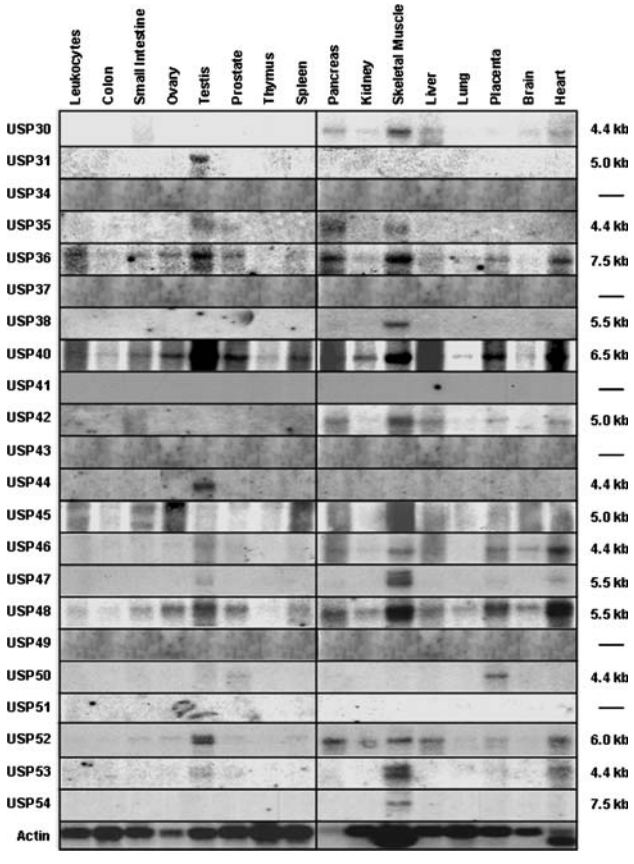

Fig. 3. Tissue distribution of the identified human USPs. Filters containing about 2 μg of polyadenylated RNAs from the indicated human tissues were hybridized with the cDNAs isolated for human USPs. RNA sizes are indicated. Filters were subsequently hybridized with a human actin probe to ascertain differences in RNA loading.

Enzymatic assays of human USPs

To analyze the enzymatic activity of the identified human USPs, isolated cDNAs for these proteins were cloned in the expression vector pGEX-3X and then co-transformed with pACY184. This vector directs the production of a ubiquitin-β-galactosidase fusion protein, which can be targeted by USPs. After induction with isopropyl-1-thio-β-D-galactopyranoside, protein extracts were analyzed by Western blot with an anti-β-galactosidase mouse monoclonal antibody. The presence of recombinant USPs was assessed by Coomassie staining of the extracts, whereas USP activity was assessed by a change in the electrophoretic mobility of the recombinant β-galactosidase, consistent with the removal of the ubiquitin moiety from the Ub-M-β-galactosidase fusion protein. As shown in Fig. 4, immunoblot analysis demonstrated that USP30, −34, −35, −36, −37, −38, −42, −44, −45, −46, −49, and −51 showed USP activity. Interestingly, USP30 and USP45 are active in this assay even though their third catalytic residue is a serine instead of the conserved Asp/Asn (Fig. 4). By contrast, USP40, −47, and −48 showed no activity in this assay, despite they possessing all the catalytic residues proposed to be important for the catalytic activity of these enzymes. On the other hand, and consistent with the lack of essential Cys, His or Asp/Asn residues in the sequence of USP50, −52, −53, and −54, these non-protease homologues of the USP family

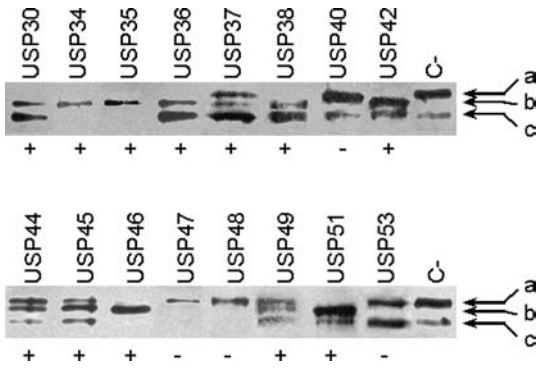

Fig. 4. Enzymatic analysis of the identified human USPs. For each experiment, XL1-Blue cells expressing a GST-USP fusion protein and Ub-M-β-gal were lysed and subjected to immunoblotting with an anti-β-gal antibody. The arrows indicate non-processed recombinant Ub-M-β-gal (a), USP-processed recombinant β-gal (b), and endogenous β-gal (c). The result of each experiment is indicated below the corresponding lane. (+), active; (−), inactive.

were completely devoid of catalytic activity against the assayed fusion protein (Fig. 4 and data not shown). Finally, the putative activity of USP31 and USP43 could not be assessed with this assay due to the lack of significant levels of both proteins using the bacterial expression system associated with the enzymatic assay employed herein.

Discussion

In addition to their classical roles in non-specific reactions of protein catabolism, proteases play essential regulatory roles in multiple biological processes through their ability to perform highly selective reactions of proteolytic processing [38]. These new regulatory functions of proteolytic enzymes are clearly evident in the growing family of USPs, whose known members are linked to a number of physiological and pathological conditions [6,14–16]. To date, 33 distinct human enzymes belonging to this protease family had been described at the amino acid sequence level and in most cases enzymatic assays had confirmed their deubiquitylating properties. However, preliminary information retrieved from databases suggested that the complexity of this protease family could be much higher than originally thought. The recent availability of the human genome sequence has opened the possibility to identify the complete repertoire of USPs produced by human cells and tissues. In this work, we report the cloning and characterization of 22 additional members of this large and complex protein family. The strategy followed to identify these novel human USPs was first based on a bioinformatic search of the human genome sequence, looking for regions with similarity to previously characterized family members. After identification of several DNA sequences encoding proteins related to USPs, and PCR amplification experiments using human cDNA libraries as template, a series of cDNAs coding for 22 distinct human proteins were finally isolated and characterized.

Overall, the structural characteristics of these new USPs are similar to those of previously described components of this family of cysteine proteinases. They contain a conserved catalytic domain with specific structural features present in these enzymes including a Cys box, an Asp-containing motif, and a His box. Furthermore, additional stretches of conserved residues are also present in the catalytic domain of all identified USPs. Consistent with these structural characteristics, functional analysis of the recombinant human USPs produced in a bacterial expression system, revealed that a number of them are active as deubiquitylating enzymes. Thus, the recombinant USP30, −34, −35, −36, −37, −38, −42, −44, −45, −46, −49, and −51 exhibit a significant proteolytic activity against a ubiquitin-β-galactosidase fusion protein commonly used to analyze the activity of these cysteine proteases [6,14–16,31]. Notably, USP30 and USP45 are also active, despite presenting a serine instead of the conserved Asp/Asn in the catalytic triad, a situation identical to that previously described for USP16 that also possesses a serine residue at the equivalent position [21]. Also consistent with the lack of essential Cys, His or Asp/Asn residues in the sequence of USP50, −52, −53 and −54, these non-protease homologues of the USP family were completely devoid of catalytic activity against the assayed fusion protein. These non-protease homologues are abundant in some protease families and may have important roles as regulatory or inhibitory molecules, by titrating protease inhibitors from the milieu or by acting as dominant negatives through their ability to bind substrates with their inactive catalytic domains [38]. It is also remarkable the absence of significant deubiquitylating activity of the recombinant catalytic domains of other USPs such as USP40, −47, and −48, which contain all characteristic structural features of these enzymes. One possibility to explain this finding is that their respective catalytic domains have diverged considerably or possess specific structural or functional constraints owing to the need to target different in vivo substrates. In fact, the finding that the mammalian USP system is composed of more than 50 distinct proteases clearly indicates that this proteolytic system has acquired a high degree of complexity during eukaryote evolution. Accordingly, some of them may target other ubiquitin-related protein modifiers as demonstrated for USP18 that specifically removes ISG15 from conjugated proteins [39], and USP21 that is a deneddylase with ability to hydrolyze Nedd8 derivatives [40]. Therefore, the observation that some USPs do not exhibit significant activity in the employed deubiquitylating assay system should not be used to rule out their relevance in this process. Interestingly, some active deubiquitylating enzymes such as human USP18 and yeast Ubp8, Ubp12, and Ubp16 have also been reported to be inactive in similar assays [39,41], providing additional evidence that these in vitro experiments may have limitations to extrapolate functional roles for human USPs. Also in this regard, it should be considered that regions distinct from the catalytic domain may influence the proteolytic efficiency of some USP family members. Detailed amino acid sequence analysis of the identified USPs revealed that a number of them have recognizable ancillary domains at the N- or C-terminal regions flanking the central catalytic domain. The most frequently detected among them is the Zn-finger ZnF-UBP domain that is found in four of the identified USPs (USP44, −45, −49, and −51). This domain is also present in some previously identified USPs and in other proteins such as histone deacetylases, and has been involved in the binding of these proteins to ubiquitin [41–43]. Likewise, the DUSP domain—which

is frequently and exclusively associated with USPs—is detected in USP48, that also contains an UBQ domain, whereas the UIM domain is found in USP37. This latter domain is also present in other USPs, as well as in several endocytic proteins such as Eps15, Eps15R, and epsins, and its presence has been found to be essential for monoubiquitylation of these proteins [44–46]. There are also ancillary domains in the identified USPs that had not been previously reported to be associated with these proteases, as is the case of the ExoIII domain found in USP52 and previously described in a variety of proteins with exonuclease activity such as ribonuclease T and the epsilon subunit of DNA polymerase III [47,48]. Structural analysis of the identified USPs also revealed the presence in some of them of archetypal sorting signals that suggested that these enzymes could be targeted to specific intracellular locations.

In this work, we have also examined the tissue distribution of the identified USPs as a preliminary step to shed some light on the physiological role of these cysteine proteases. This analysis revealed that some family members such as USP36, −40, −42, −46, −48, and −52 are widely expressed in a variety of human tissues, suggesting that they participate in general deubiquitylating reactions which may occur at basal levels in all tissues. By contrast USP30, −31, −35, −38, −44, −45, −47, −50, −53, and −54 are only produced at significant levels by one or a few tissues. Interestingly, many of these USPs that exhibit a restricted expression pattern are detected in skeletal muscle or testis, indicating that USP-mediated functions may be especially relevant in these tissues. This finding is also of particular interest in light of previous data reporting the association of ubiquitin-processing abnormalities in pathological conditions involving skeletal muscle and testis [49,50]. These putative associations between USPs and skeletal muscle or reproductive diseases may also imply the possibility that inherited alterations in these genes could be linked to familial forms of these pathologies.

Chromosomal location analysis of USP genes indicates that they are not clustered in the human genome, a situation distinct from that observed in other densely populated protease families whose encoding genes are significantly clustered in mammalian genomes [29]. Genetic lesions in the regions in which some of the identified USP genes are located have been linked to several diseases whose responsible genes remain to be characterized. This is the case of a spinal muscular atrophy linked to 12q23-24 [47], the same region containing USP30, an USP predominantly expressed in human skeletal muscle. Likewise, the testis-specific USP44 is located at 12q22 in a region commonly deleted in human male germ cell tumors [51]. Hereditary X-linked diseases in the region to which USP51 is localized (Xp11), and involving muscular atrophy or mental retardation syndromes have also been reported [52,53]. It will be of future interest to examine the possibility that these USP genes could be a target of some of these genetic abnormalities. Likewise, the finding that with the single exception of USP41, all human USPs identified in this work have conserved orthologues in the mouse genome opens the possibility to generate mice deficient in those genes of major interest, an aspect that could contribute to clarify the role of this proteolytic system in physiological and pathological conditions. In this regard, it is interesting that the two available mutant mice deficient in specific USPs exhibit marked phenotype abnormalities [28,49], suggesting that beyond the apparent redundancy in this large proteolytic system, there are specific functions performed by these enzymes in mammalian tissues resulting from their ability to target-specific substrates [22,25]. It will be also of great interest to examine the possibility that some of the identified USPs may play specific roles in tumor development and progression, in a similar way to that reported for other family members whose unregulated expression or activity have been linked to cancer [17–20].

In summary, the identification and cloning of 22 additional members of the USP family of cysteine proteases emphasizes the relevance of the protein modification reactions mediated by these enzymes in human tissues. According to our exhaustive analysis of the human genome, the total number of family members would be of 55, including five non-protease homologues: USP39, −50, −52, −53, and 54. The complexity of the system would be even higher if we consider that the multiple copies of USP17 and USP17L encoded by tandem repetitive sequences present in the human genome [54] have been annotated as single USP family members [29]. Further studies directed to analyze the functional roles of the identified USPs, including our ongoing project of generation of mutant mice deficient in selected family members, will be necessary to clarify the relative relevance of the individual components of this complex family of proteolytic enzymes.

Acknowledgments

We thank Drs. L.M. Sánchez, X. S. Puente, G. Velasco, M. Balbín, A.M. Pendás, and J.M.P. Freije for helpful comments, Drs. M. Hochstrasser and G. Marfany for providing reagents, and M. Fernández for excellent technical assistance.

References

[1] M. Hochstrasser, Evolution and function of ubiquitin-like protein-conjugation systems, Nat. Cell Biol. 2 (2000) E153–157.
[2] A.M. Weissman, Themes and variations on ubiquitylation, Nat. Rev. Mol. Cell Biol. 2 (2001) 169–178.
[3] M.H. Glickman, A. Ciechanover, The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction, Physiol. Rev. 82 (2002) 373–428.

[4] C.M. Pickart, Mechanisms underlying ubiquitination, Annu. Rev. Biochem. 70 (2001) 503–533.
[5] Y. Ohsumi, Molecular dissection of autophagy: two ubiquitin-like systems, Nat. Rev. Mol. Cell Biol. 2 (2001) 211–216.
[6] K.D. Wilkinson, Regulation of ubiquitin-dependent processes by deubiquitinating enzymes, FASEB J. 11 (1997) 1245–1256.
[7] E.T. Yeh, L. Gong, T. Kamitani, Ubiquitin-like proteins: new wines in new bottles, Gene 248 (2000) 1–14.
[8] T. Gan-Erdene, K. Nagamalleswari, L. Yin, K. Wu, Z.Q. Pan, K.D. Wilkinson, Identification and characterization of DEN1, a deneddylase of the ULP family, J. Biol. Chem. 278 (2003) 28892–28900.
[9] G. Mariño, J.A. Uría, X.S. Puente, V. Quesada, J. Bordallo, C. López-Otín, Human autophagins, a family of cysteine proteinases potentially implicated in cell degradation by autophagy, J. Biol. Chem. 278 (2003) 3671–3678.
[10] M.Y. Balakirev, S.O. Tcherniuk, M. Jaquinod, J. Chroboczek, Otubains: a new family of cysteine proteases in the ubiquitin pathway, EMBO Rep. 4 (2003) 517–522.
[11] P.C. Evans, T.S. Smith, M.J. Lai, M.G. Williams, D.F. Burke, K. Heyninck, M.M. Kreike, R. Beyaert, T.L. Blundell, P.J. Kilshaw, A novel type of deubiquitinating enzyme, J. Biol. Chem. 278 (2003) 23180–23186.
[12] B. Burnett, F. Li, R.N. Pittman, The polyglutamine neurodegenerative protein ataxin-3 binds polyubiquitylated proteins and has ubiquitin protease activity, Hum. Mol. Genet. (2003).
[13] R. Verma, L. Aravind, R. Oania, W.H. McDonald, J.R. Yates 3rd, E.V. Koonin, R.J. Deshaies, Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome, Science 298 (2002) 611–615.
[14] A. D'Andrea, D. Pellman, Deubiquitinating enzymes: a new class of biological regulators, Crit. Rev. Biochem. Mol. Biol. 33 (1998) 337–352.
[15] C.H. Chung, S.H. Baek, Deubiquitinating enzymes: their diversity and emerging roles, Biochem. Biophys. Res. Commun. 266 (1999) 633–640.
[16] S.S. Wing, Deubiquitinating enzymes—the importance of driving in reverse along the ubiquitin–proteasome pathway, Int. J. Biochem. Cell. Biol. 35 (2003) 590–605.
[17] K. Gupta, M. Chevrette, D.A. Gray, The Unp proto-oncogene encodes a nuclear protein, Oncogene 9 (1994) 1729–1731.
[18] F.R. Papa, M. Hochstrasser, The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene, Nature 366 (1993) 313–319.
[19] Y. Zhu, K. Lambert, C. Corless, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, A.D. D'Andrea, DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes, J. Biol. Chem. 272 (1997) 51–57.
[20] S. Naviglio, C. Mattecucci, B. Matoskova, T. Nagase, N. Nomura, P.P. Di Fiore, G.F. Draetta, UBPY: a growth-regulated human ubiquitin isopeptidase, EMBO J. 17 (1998) 3241–3250.
[21] S.Y. Cai, R.W. Babbitt, V.T. Marchesi, A mutant deubiquitinating enzyme (Ubp-M) associates with mitotic chromosomes and blocks cell division, Proc. Natl. Acad. Sci. USA 96 (1999) 2828–2833.
[22] M. Li, D. Chen, A. Shiloh, J. Luo, A.Y. Nikolaev, J. Qin, W. Gu, Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization, Nature 416 (2002) 648–653.
[23] D. Moazed, D. Johnson, A deubiquitinating enzyme interacts with SIR4 and regulates silencing in *S. cerevisiae*, Cell 86 (1996) 667–677.
[24] Y. Huang, R.T. Baker, J.A. Fischer-Vize, Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene, Science 270 (1995) 1828–1831.
[25] C. Curcio-Morelli, A.M. Zavacki, M. Christofollete, B. Gereben, B.C. de Freitas, J.W. Harney, Z. Li, G. Wu, A.C. Bianco, Deubiquitination of type 2 iodothyronine deiodinase by von Hippel–Lindau protein-interacting deubiquitinating enzymes regulates thyroid hormone activation, J. Clin. Invest. 112 (2003) 189–196.
[26] Z. Li, X. Na, D. Wang, S.R. Schoen, E.M. Messing, G. Wu, Ubiquitination of a novel deubiquitinating enzyme requires direct binding to von Hippel–Lindau tumor suppressor protein, J. Biol. Chem. 277 (2002) 4656–4662.
[27] M. Pantaleon, M. Kanai-Azuma, J.S. Mattick, K. Kaibuchi, P.L. Kaye, S.A. Wood, FAM deubiquitylating enzyme is essential for preimplantation mouse embryo development, Mech. Dev. 109 (2001) 151–160.
[28] K.J. Ritchie, M.P. Malakhov, C.J. Hetherington, L. Zhou, M.T. Little, O.A. Malakhova, J.C. Sipe, S.H. Orkin, D.E. Zhang, Dysregulation of protein modification by ISG15 results in brain cell injury, Genes Dev. 16 (2002) 2207–2212.
[29] X.S. Puente, L.M. Sánchez, C.M. Overall, C. López-Otín, Human and mouse proteases: a comparative genomic approach, Nat. Rev. Genet. 4 (2003) 544–558.
[30] X.S. Puente, C. López-Otín, A genomic analysis of rat proteases and protease inhibitors, Genome. Res. (2004), in press.
[31] R.T. Baker, J.W. Tobias, A. Varshavsky, Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. Cloning of UBP2 and UBP3 and functional analysis of the UBP gene family, J. Biol. Chem. 267 (1992) 23364–23375.
[32] C.A. Paulding, M. Ruvolo, D.A. Haber, The Tre2 USP6 oncogene is a hominoid-specific gene, Proc. Natl. Acad. Sci. USA 100 (2003) 2507–2511.
[33] Z. Lygerou, G. Christophides, B. Seraphin, A novel genetic screen for snRNP assembly factors in yeast identifies a conserved protein, Sad1p, also required for pre-mRNA splicing, Mol. Cell Biol. 19 (1999) 2008–2020.
[34] A. Amerik, S. Swaminathan, B.A. Krantz, K.D. Wilkinson, M. Hochstrasser, In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome, EMBO J. 16 (1997) 4826–4838.
[35] M. Hu, P. Li, M. Li, W. Li, T. Yao, J.W. Wu, W. Gu, R.E. Cohen, Y. Shi, Crystal structure of a UBP-family deubiquitinating enzyme in isolation and in complex with ubiquitin aldehyde, Cell 111 (2002) 1041–1054.
[36] R. Menard, J. Carriere, P. Laflamme, C. Plouffe, H.E. Khouri, T. Vernet, D.C. Tessier, D.Y. Thomas, A.C. Storer, Contribution of the glutamine 19 side chain to transition-state stabilization in the oxyanion hole of papain, Biochemistry 30 (1991) 8924–8928.
[37] S.C. Johnston, C.N. Larsen, W.J. Cook, K.D. Wilkinson, C.P. Hill, Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 A resolution, EMBO J. 16 (1997) 3787–3796.
[38] C. López-Otín, C.M. Overall, Protease degradomics: a new challenge for proteomics, Nat. Rev. Mol. Cell Biol. 3 (2002) 509–519.
[39] M.P. Malakhov, O.A. Malakhova, K.I. Kim, K.J. Ritchie, D.E. Zhang, UBP43 (USP18) specifically removes ISG15 from conjugated proteins, J. Biol. Chem. 277 (2002) 9976–9981.
[40] L. Gong, T. Kamitani, S. Millas, E.T. Yeh, Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins, J. Biol. Chem. 275 (2000) 14212–14216.
[41] A.Y. Amerik, S.J. Li, M. Hochstrasser, Analysis of the deubiquitinating enzymes of the yeast *Saccharomyces cerevisiae*, Biol. Chem. 381 (2000) 981–992.
[42] D. Seigneurin-Berny, A. Verdel, S. Curtet, C. Lemercier, J. Garin, S. Rousseaux, S. Khochbin, Identification of components of the murine histone deacetylase 6 complex: link between acetylation and ubiquitination signaling pathways, Mol. Cell. Biol. 21 (2001) 8035–8044.
[43] S.S. Hook, A. Orian, S.M. Cowley, R.N. Eisenman, Histone deacetylase 6 binds polyubiquitin through its zinc finger PAZ

domain and copurifies with deubiquitinating enzymes, Proc. Natl. Acad. Sci. USA 99 (2002) 13425–13430.
[44] P.P. Di Fiore, S. Polo, K. Hofmann, When ubiquitin meets ubiquitin receptors: a signalling connection, Nat. Rev. Mol. Cell Biol. 4 (2003) 491–497.
[45] L. Hicke, Protein regulation by monoubiquitin, Nat. Rev. Mol. Cell Biol. 2 (2001) 195–201.
[46] E. Klapisz, I. Sorokina, S. Lemeer, M. Pijnenburg, A.J. Verkleij, P.M. van Bergen en Henegouwen, A ubiquitin-interacting motif (UIM) is essential for Eps15 and Eps15R ubiquitination, J. Biol. Chem. 277 (2002) 30746–30753.
[47] I.V. Shevelev, U. Hubscher, The 3' 5' exonucleases, Nat. Rev. Mol. Cell Biol. 3 (2002) 364–376.
[48] A.J. van der Vleuten, C.M. van Ravenswaaij-Arts, C.J. Frijns, A.P. Smits, G. Hageman, G.W. Padberg, H. Kremer, Localisation of the gene for a dominant congenital spinal muscular atrophy predominantly affecting the lower limbs to chromosome 12q23-q24, Eur. J. Hum. Genet. 6 (1998) 376–382.
[49] S.M. Wilson, B. Bhattacharyya, R.A. Rachel, V. Coppola, L. Tessarollo, D.B. Householder, C.F. Fletcher, R.J. Miller, N.G. Copeland, N.A. Jenkins, Synaptic defects in ataxia mice result from a mutation in Usp14, encoding a ubiquitin-specific protease, Nat. Genet. 32 (2002) 420–425.
[50] C. Sun, H. Skaletsky, B. Birren, K. Devon, Z. Tang, S. Silber, R. Oates, D.C. Page, An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y, Nat. Genet. 23 (1999) 429–432.
[51] V.V. Murty, B. Renault, C.T. Falk, G.J. Bosl, R. Kucherlapati, R.S. Chaganti, Physical mapping of a commonly deleted region, the site of a candidate tumor suppressor gene, at 12q22 in human male germ cell tumors, Genomics 35 (1996) 562–570.
[52] H. Kobayashi, L. Baumbach, T.C. Matise, A. Schiavi, F. Greenberg, E.P. Hoffman, A gene for a severe lethal form of X-linked arthrogryposis (X-linked infantile spinal muscular atrophy) maps to human chromosome Xp11.3-q11.2, Hum. Mol. Genet. 4 (1995) 1213–1216.
[53] J. Chelly, Breakthroughs in molecular and cellular mechanisms underlying X-linked mental retardation, Hum. Mol. Genet. 8 (1999) 1833–1838.
[54] T. Okada, Y. Gondo, J. Goto, I. Kanazawa, S. Hadano, J.E. Ikeda, Unstable transmission of the RS447 human megasatellite tandem repetitive sequence that contains the USP17 deubiquitinating enzyme gene, Hum. Genet. 110 (2002) 302–313.