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Ras–GAP SH3 domain binding protein (G3BP) is a modulator of USP10, a novel human ubiquitin specific protease

Chiara Soncini[1,2], Ingrid Berdo[1] and Giulio Draetta*[,1]

[1]*European Institute of Oncology, Department of Experimental Oncology, Via Ripamonti 435, 20141 Milan, Italy*

Degradation of cellular proteins through ubiquitination is a fundamental strategy for regulating biological pathways. De-ubiquitination, i.e. the removal of ubiquitin from proteins and peptides to which ubiquitin is attached, is catalyzed by processing proteases known as de-ubiquitinating enzymes. We are studying the biology of a family of de-ubiquitinating enzymes, the mammalian ubiquitin-specific proteases (USPs), some of which appear to play a role in growth control. Given the fact that the modes of regulation of USPs and of their substrate specificity are poorly understood, we decided to attempt the identification of USP interacting proteins. Using the yeast two-hybrid system (2HS), we have isolated a cDNA clone whose product specifically interacts with USP10 but not with other USP baits tested. The isolated clone encodes a protein known to interact with the Ras-GTPase activating protein (G3BP). This interaction was further confirmed by performing a 2HS with G3BP, which led to the isolation of USP10 encoding cDNAs. We validated the interaction between the two proteins by performing *in vitro* binding assays and immunoprecipitations in human cells. G3BP does not appear to be a substrate of USP10; it rather inhibits the ability of USP10 to disassemble ubiquitin chains. The USP10/G3BP complex appears to co-immunoprecipitate with ubiquitinated species that could be substrates of USP10. *Oncogene* (2001) **20,** 3869–3879.

Keywords: de-ubiqitinating enzyme; G3BP; ubiquitin

Introduction

Several important regulatory and structural proteins are subject to modification by the attachment of ubiquitin or ubiquitin like proteins. The specific attachment of ubiquitin molecules to a particular substrate is highly regulated. Ubiquitination is achieved through a cascade of reactions that involve first a ubiquitin activating enzyme (E1), which in the presence of ATP is linked to ubiquitin through the formation of a thioester bond. Ubiquitin is then transferred to a ubiquitin conjugating enzyme (E2). The specific targeting of a protein substrate is then achieved through its interaction with both a specific E2 and a ubiquitin ligase (E3), leading to the formation of isopeptide bonds. In yeast, an E4 function has also been described, which might have the role of extending the ubiquitin trees (Koegl *et al*., 1999). The degradation steps that follow protein ubiquitination occur on the proteasome. The 26S proteasome will recognize the ubiquitinated target and will induce its degradation through an ATP dependent unfolding process. Ubiquitination also acts as a targeting signal, delivering the modified protein to different locations in the cell and potentially modifying its activity, interactions or half-life (for review, see Hershko and Ciechanover, 1998; Laney and Hochstrasser, 1999).

Hydrolysis of ubiquitin conjugates is needed to regenerate free ubiquitin from branched ubiquitin generated post-degradation, but also to generate free ubiquitin subunits from the synthesized linear poly-ubiquitin chains or from ubiquitin fusion proteins. It has also been hypothesized that protein ubiquitination can be a reversible process and that specific substrates can be de-ubiquitinated by USPs. The existence of active de-ubiquitinating activities is also supported by the finding that inhibition of the proteolytic activity of the proteasome, while inducing the accumulation of protein substrates does not result in an increase in their overall ubiquitination.

De-ubiquitination is catalyzed by specialized thiol proteases known as de-ubiquitinating enzymes (for a recent review see Ciechanover *et al*., 2000). These enzymes hydrolyze the amide bond between the C-terminal glycine residue of ubiquitin and primary amino groups of the substrate protein. More than 60 de-ubiquitinating enzymes have been identified thus far in genome databases, and they are commonly divided in two distinct families: Ubiquitin C-terminal Hydrolases (UCH) and Ubiquitin-Specific Proteases (USPs) (D'Andrea and Pellman, 1998; Wilkinson, 1997; Wilkinson and Hochstrasser, 1998). We are interested in studying the biology and biochemistry of mammalian USPs, also known as UBPs (ubiquitin isopeptidases), they presently form the largest enzyme family in the ubiquitin system and are characterized by a core region containing conserved regions, the Cys and His

*Correspondence: G Draetta; E-mail: gdraetta@ieo.it
[2]Current address: Pharmacia Corporation, Discovery Research Oncology, Nerviano, Italy 20014
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boxes (Baker *et al*., 1992), surrounded by divergent sequences, most commonly at the N-terminal end. The N-terminal end is likely responsible for enzyme specificity and/or for controlling subcellular localization (Lin *et al*., 2000). In addition, there are indications that distinct USPs could have specialized functions.

Genetic investigations in yeast and in *Drosophila* have identified certain USPs as critical regulators of biological processes such as ubiquitin homeostasis (Papa and Hochstrasser, 1993; Swaminathan *et al*., 1999), gene silencing (Moazed and Johnson, 1996) and development (Huang *et al*., 1995). Genetic evidence would in fact indicate that mutation in selected USP genes can affect a given biological pathway (Cadavid *et al*., 2000). The specific phenotypes resulting from the disruption of genes encoding such USPs demonstrated that USPs could have a high degree of substrate specificity (Moazed and Johnson, 1996; Huang and Fischer-Vize, 1996).

USP deregulation has been detected in cancer. It has been shown that overexpression of a mouse cDNA encoding Unp (USP4) leads to oncogenic transformation of NIH3T3 cells (Gupta *et al*., 1993). The human Unp mRNA levels were found to be elevated in small cell lung carcinomas and adenocarcinomas of the lung, suggesting its involvement in tumorigenesis (Gray *et al*., 1995), although a study describes results that are inconsistent with the previous findings (Frederick *et al*., 1998). Recently, a cDNA encoding Dub-1, a mouse USP, was found in the course of a screen for cytokine regulated genes. Dub-1 overexpression results in the induction of growth arrest, leading to the suggestion that it might have growth suppressive ability *in vivo* (Zhu *et al*., 1996). The same group isolated a highly related protein Dub-2, that is specifically induced by IL-2 (Zhu *et al*., 1997). We have previously characterized a novel human USP, USP8 (UBPY) which has de-ubiquitinating activity *in vitro* and *in vivo*, and we have shown that microinjection of anti-USP8 prevents fibroblasts from entering S phase in response to serum stimulation (Naviglio *et al*., 1998). Two additional USPs have recently also been identified and characterized *in vitro* (Baker *et al*., 1999; Sloper-Mould *et al*., 1999).

We used a two-hybrid screen to isolate potential USP interactors. Here we demonstrate that USP10 is a de-ubiquitinating enzyme and that it interacts *in vitro* and *in vivo* with G3BP, a protein involved in the Ras signal transduction pathway (Parker *et al*., 1996). G3BP does not appear to be a substrate of USP10; it rather inhibits USP10 activity. Our data demonstrate that the two proteins are part of a complex that likely regulates USP10 function *in vivo*.

Results

USP10 cloning and characterization

A cDNA encoding a potential USP was found in GenBank (accession number D80012). Using the first 1200 bp of this cDNA (a kind gift from N Nomura) as a probe, we screened a human cDNA library to identify any potentially longer clone. All the positive clones obtained from the screen were found to have the same DNA sequence and a 5′ in-frame stop codon. The third ATG in the clone is likely the starting codon having the strongest Kozac consensus; it is also conserved in a highly similar mouse cDNA (GenBank accession number D84096). The identified ORF encodes a protein of 848 amino acid residues with a predicted MW of 92 900 Da. It contains regions matching the consensus cysteine and histidine boxes typical of the USP family of de-ubiquitinating enzymes (Figure 1a). According to the nomenclature proposed by the Human Genome Organization (HUGO) Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature), we are calling the encoded protein USP10.

Figure 1b shows the characterization of an anti USP10 polyclonal antiserum. The antiserum recognized a band of about 100 kDa in a cell lysate. *In vitro* translated protein was also recognized by the antiserum and it comigrated with the band detected in the cell lysate. USP10 was ubiquitously expressed both at the RNA and at the protein level, with increased abundance in transformed cells compared to normal ones (C Soncini, Maria Capra and GF Draetta, 2001, unpublished).

We then expressed USP10 as a glutathione S-transferase (GST) fusion protein in *E. coli* and after purifying it on a glutathione-Sepharose column, assayed its de-ubiquitinating activity using a previously characterized artificial USP substrate, an HA-tagged ubiquitin hexamer (HA-Ub6) (Naviglio *et al*., 1998). It has been demonstrated that such epitope-tagged ubiquitin can be conjugated correctly *in vivo* and *in vitro* to cellular proteins which then become targets of proteolytic cleavage by the proteasome (Ellison and Hochstrasser, 1991; Treier *et al*., 1994; Hateboer *et al*., 1996; Diehl *et al*., 1997). We could not detect any activity under these conditions. On the contrary, upon expressing the putative USP10 catalytic domain as GST-fusion protein in bacteria, we could readily detect activity.

We wanted to exclude the possibility that lack of activity in the full-length USP10 protein might be due to misfolding or lack of post-translational modifications. To this end, we generated a His6-tagged full-length wild-type construct and one carrying a Cys-to-Ala mutation in the putative active site cysteine. These were expressed in baculovirus-infected insect cells, purified and tested for activity in the assay. Indeed, upon incubating Ha-Ub6 with increasing amount of purified baculovirus, expressed full-length wild-type USP10 we could readily detect activity (Figure 2). When assayed under the same conditions, the protein mutated in the putative active site cysteine was inactive. Degradation was dose dependent and completely abolished by the addition of 5 mM N-ethyl maleimide (NEM), a known inhibitor of de-ubiquitinating enzymes (Stein *et al*., 1995; Wilkinson *et al*.,

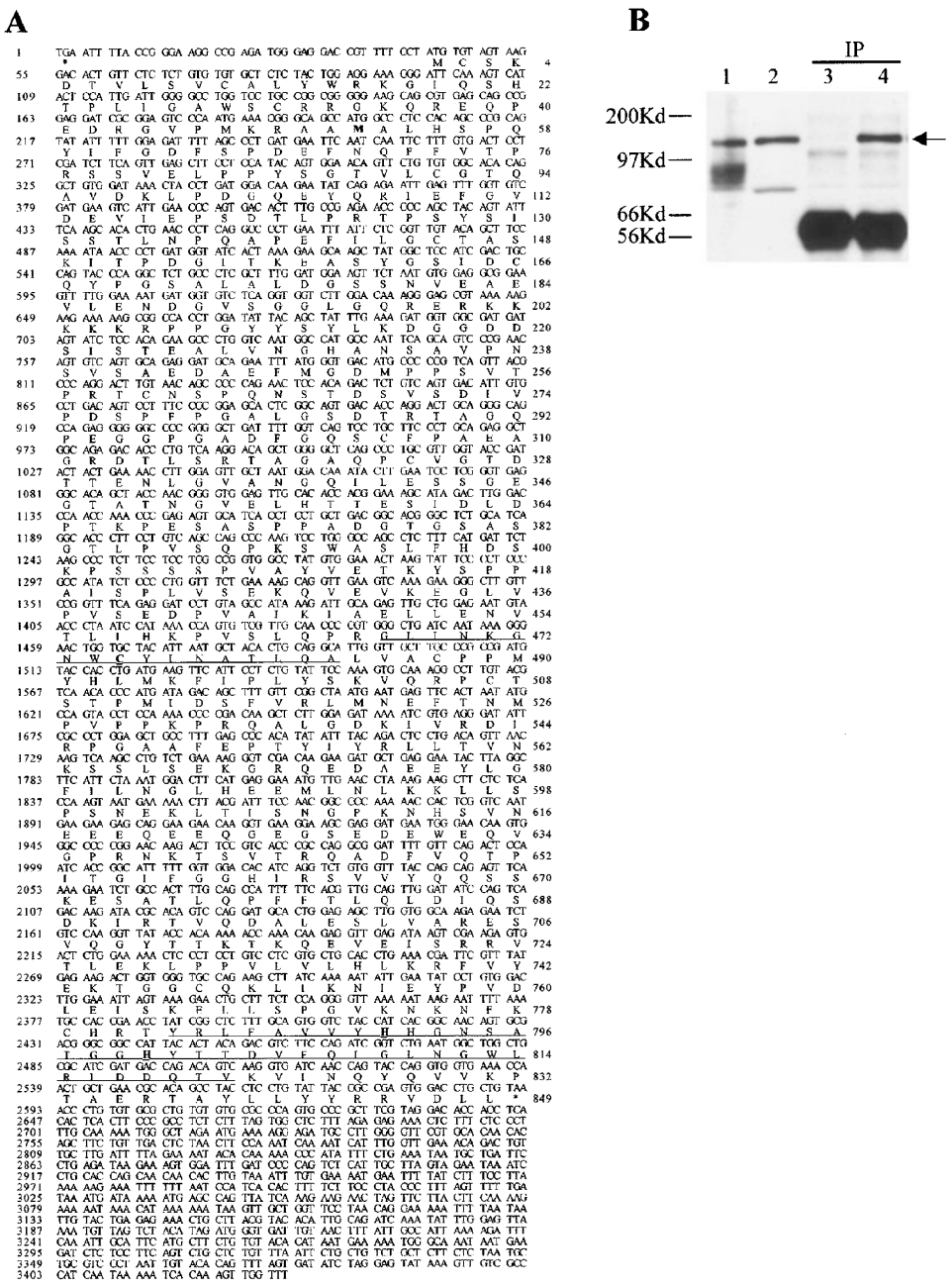

Figure 1 (**a**) Human USP10. DNA sequence of the human USP10 cDNA with nucleotide numbers indicated to the left. Also shown is USP10 open reading frame with amino acid residues numbered to the right. Underlined are the Cys and His boxes and bold the putative first methionine. (**b**) USP10 antibody characterization. Immunoblot analysis with anti USP10 polyclonal antibody on *in vitro* translated product (1), U2OS total lysate (2), U2OS lysate immunoprecipitated with preimmune serum (3) and anti USP10 (4)

1995) (Figure 2). We obtained similar results using USP10 immunoprecipitated from human cell extracts (not shown).

USP10 activity could also be detected using a ubiquitin isopeptide dimer as a substrate (Figure 3a). We found that overexpression of wild-type USP10 in cells transfected with HA-Ub caused a decrease in protein ubiquitination (Figure 3b). In the same experiment, a construct encoding a Cys to Ala mutant was completely inactive (Figure 3b). This demonstrated that USP10 overexpression can effectively drive protein de-ubiquitination.

G3BP is a USP10 interacting protein

To identify potential USP10 substrates and/or modulators of its function, we performed a yeast two-hybrid screen. The bait was constructed to express a fusion protein between the LexA DNA binding domain (amino acids 1–211) and the full-length USP10 cDNA.

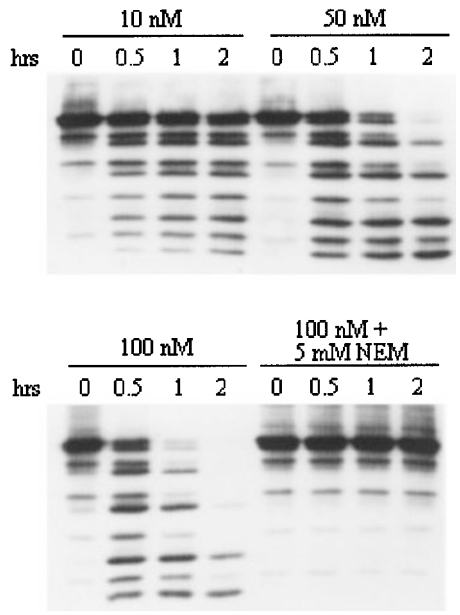

Figure 2 USP10 de-ubiquitinating activity on linear ubiquitin. Increasing amount of purified Bac-USP10 were incubated with Ha-Ub6mer. Samples were collected at different time point and loaded on a SDS–PAGE. Western blot was performed with an anti-HA antibody. One time course was done in the presence of 5 mM N-ethylmaleimide (NEM)

We screened both a mouse embryo and a human lymphocyte cDNA library. Two highly similar cDNA clones were identified in both libraries, which were also the most represented among the positives obtained from either screen. The cDNAs were found to encode *G*AP SH*3*-*b*inding *p*rotein (G3BP), a protein previously characterized as binding the SH3 domain of Ras–*G*TPase *A*ctivating *p*rotein (Ras–GAP) (Parker *et al*., 1996). By direct two-hybrid, we found that the interaction occurred only with USP10 (either the wild-type protein or a Cys-to-Ala mutant) but not with two other USPs used as baits. Along with USP10, we tested USP8 (UBPY) (Naviglio *et al*., 1998) and USP4 (UNPH) (Frederick *et al*., 1998; Gray *et al*., 1995). Neither of the two interacted with G3BP in this system. The specificity of this interaction was also demonstrated by the fact that a region comprising the amino terminus of USP10, which does not contain the catalytic site and is poorly conserved between the different UBPs, was sufficient for USP10 interaction with G3BP (Figure 4a).

We also performed a two-hybrid screen with the G3BP full-length cDNA as a bait, and identified among 20 clones a clone encoding amino acids 52–280 of USP10 (Figure 4b). This result confirmed the specificity of the detected interaction between G3BP and USP10 even further and prompted us to continue to investigate its role. We then set-up a binding assay using *in vitro* translated G3BP and the GST–UBPO

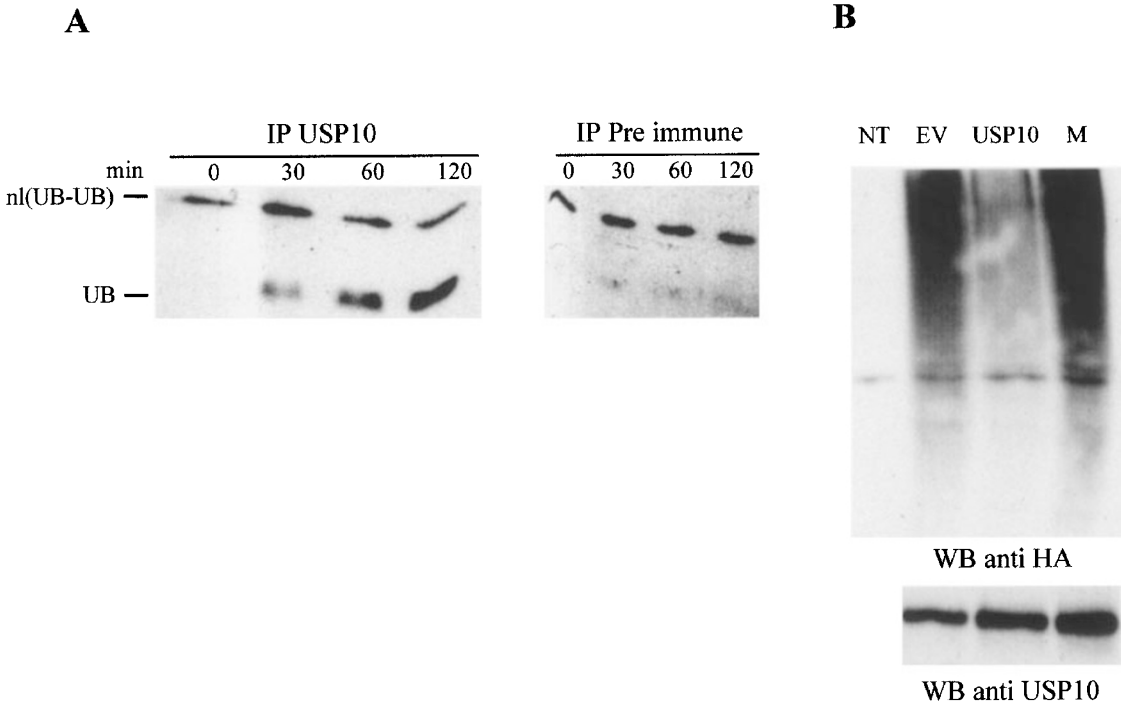

Figure 3 USP10 de-ubiquitinating activity. (**a**) Non linear ubiquitin was incubated with USP10 immunoprecipitated from cell lysate. Samples were collected at different time point and loaded on a SDS–PAGE. Western blot was performed with an anti ubiquitin antibody. IP with preimmune serum was used as negative control. (**b**) U2OS cells were co-transfected with 10 μg of HA-Ub plasmid and 10 μg of pCMV empty vector (EV), pCMV–USP10 wild-type Cys to Ala mutant (M). Lysates were subjected to SDS–PAGE (8%) and to anti-USP10 or anti-HA immunoblotting

protein. Figure 5a shows that G3BP could interact with USP10 *in vitro*; the GST moiety alone did not appear to interact significantly with G3BP. Similar results were obtained upon using GST–G3BP and *in vitro* translated USP10 (not shown).

To test whether this interaction occurs in living cells we performed a co-immunoprecipitation experiment. A lysate obtained from the human osteosarcoma cell line U2OS was incubated with either an anti-USP10 monoclonal antibody or with an anti-G3BP polyclonal antiserum, and immunoprecipitations were performed followed by immunoblotting with either antibody. As shown in Figure 5b, we could demonstrate that the two proteins interact in intact cells. The specificity of the interaction between USP10 and G3BP was also demonstrated by the absence of a signal using an anti-USP8 monoclonal antibody or a preimmune serum. We obtained the same result using lysates from different cell lines. The interaction between the two proteins appeared to be stable and we could detect it both in growing and quiescent (serum starved) human fibroblasts. In comparison, G3BP only appears to interact with Ras–GAP in growing but not in quiescent cells (Gallouzi *et al*., 1998).

USP10 and G3BP are part of a high molecular weight complex

To explore the hypothesis that USP10 and G3BP could be part of larger complex that might even contain USP10 substrates, we analysed protein complexes after treatment with the chemical cross-linker EDC (see Materials and methods). Similar experiments were performed by Gallouzi *et al*. (1998) to show a direct interaction between RasGAP and G3BP. As shown in Figure 6, immunoblotting with either anti-G3BP or anti-USP10 antibodies of cross-linked lysates revealed high Mr. immunoreactive bands. Using an anti-G3BP antibody on the cross-linked lysate we noticed a band of approximately 180 kDa that could correspond to the RasGAP–G3BP complex described by Gallouzi *et al*. (1998); in the same lane the complete disappearance of the G3BP band was also evident (Figure 6a). We were also able to immunoprecipitate the higher band with both anti-G3BP and anti-USP10 antibodies (Figure 6a). In our experimental conditions this band was not recognized by an anti RasGAP antibody. This high molecular weight complex must obviously contain

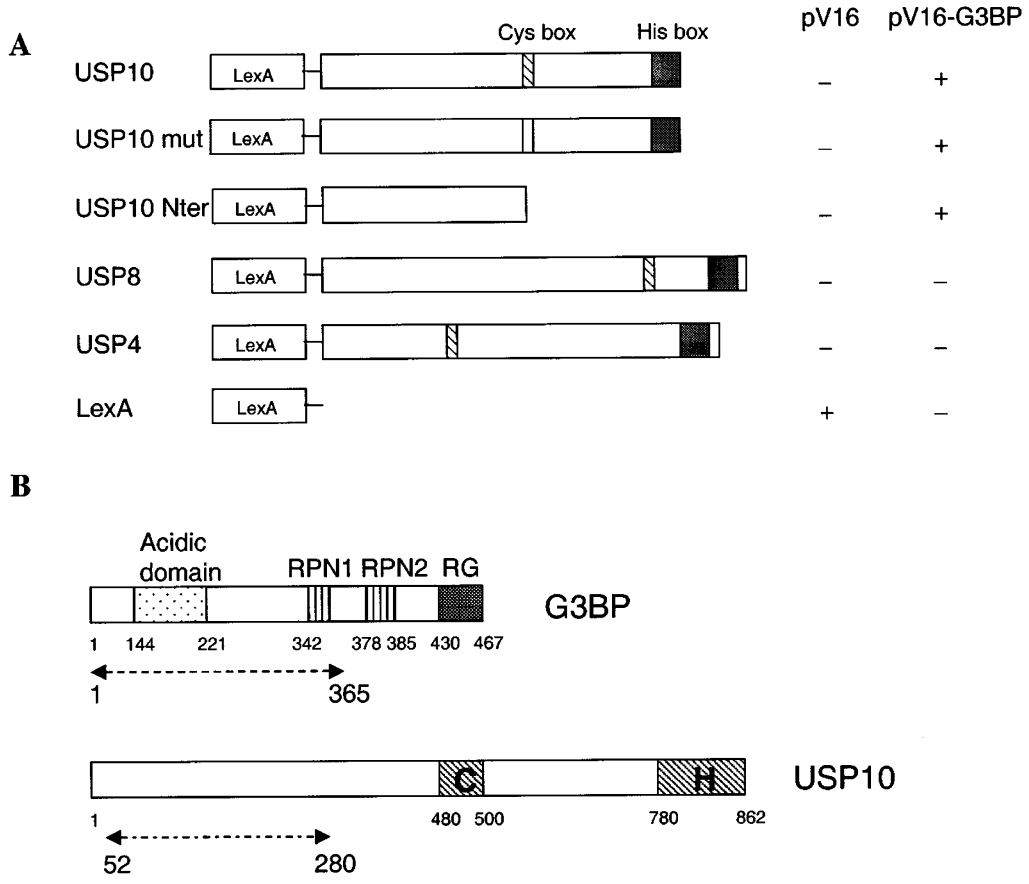

Figure 4 Schematic representation of the LexA fusion proteins used and two-hybrid assay. (**a**) The *S. cerevisiae* strain L40 was transformed with the indicated pLexA and pV16 plasmids and β-Galactosidase activity (average of three independent transformations) was measured by a filter assay for strains expressing the indicated fusion proteins. (**b**) Schematic representation of G3BP and USP10. Arrows below the schema indicate the longer insert fished out by two-hybrid with USP10 (- - -) and G3BP (-.-). RNP, RNA binding motif; RG, arginine glycine rich region; C, cysteine box; H, histidine box

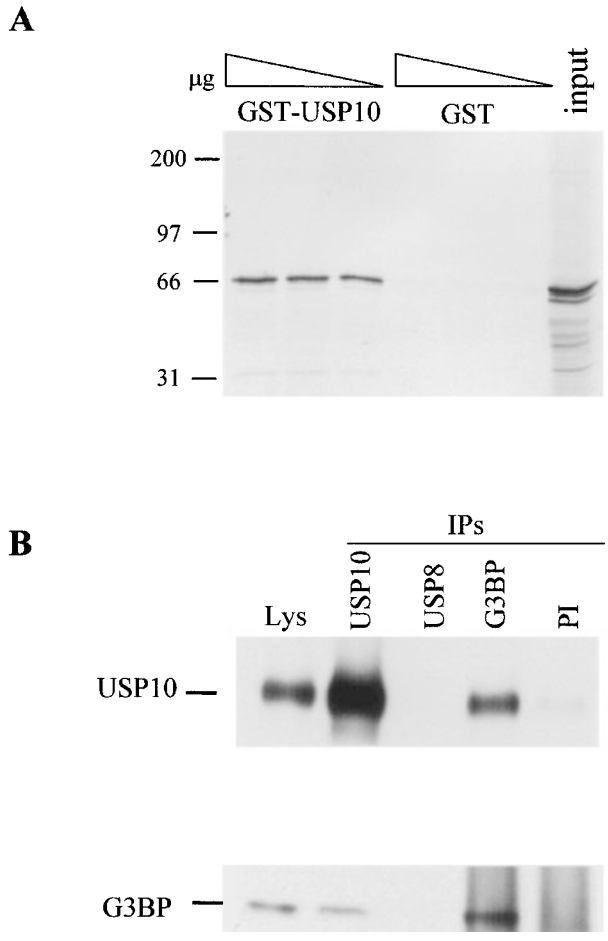

Figure 5 USP10 complex with G3BP. (**a**) *In vitro* binding assay. Increasing amount of GST and GST–USP10 fusion proteins were incubated with the *in vitro* translation product of G3BP as described in Materials and methods. The protein complexes were separated on a 10% SDS polyacrylamide gel. Two μl of the *in vitro* translation reaction is shown as control. (**b**) Co-immunoprecipitation experiment. U2OS cell lysate was incubated with anti USP10 monoclonal antibody or anti G3BP polyclonal antibody and Western blotted with both antibodies. USP8 monoclonal antibody and a polyclonal preimmune serum were used as control

USP10 and G3BP, and possibly other, yet unidentified, components.

Association of G3BP and USP10 with ubiquitinated species

We tested whether G3BP is a substrate of G3BP. After treating human U2OS or HeLa cells with the proteasome inhibitor MG132, we prepared cell lysates and performed immunoblotting with anti-G3BP antibody. The protein level remained constant (Figure 7a) whereas the level of β-catenin (which is known to be turned over rapidly through ubiquitination and proteasome dependent degradation (see Maniatis, 1999 for review)) increased substantially as a result of the treatment. This allows us to conclude that under the tested conditions G3BP is quite stable and unlikely to be degraded through the ubiquitin–proteasome pathway. Upon immunoprecipitation of lysates from cells transfected with a plasmid expressing HA-tagged ubiquitin and treated with MG132, with the anti-G3BP antibody we could detect slow migrating bands typical of ubiquitinated proteins (Figure 7b). These bands were detected by immunoblotting with an anti-HA antibody. Similar results were obtained by treating non-transfected cells with MG132 and immunoblotting with an anti-ubiquitin antibody (not shown). Interestingly, transfection of a USP10 cDNA, but not of a USP8 or 4 construct, was able to inhibit the accumulation of these ubiquitin-containing bands (not shown), suggesting that USP10 could be the specific enzyme regulating this reaction.

To verify whether the ubiquitinated bands associated with G3BP were due to G3BP ubiquitination, we performed an immunoprecipitation from a cell lysate subjected to a denaturing treatment with SDS (see Materials and methods). As shown in Figure 8, ubiquitin-containing bands disappeared upon treatment, indicating the possibility that ubiquitinated proteins other than G3BP can interact with G3BP and USP10.

To test whether G3BP could alter USP10 activity we performed an *in vitro* de-ubiquitination assay in the presence of G3BP. HA-Ub6 substrate was incubated with recombinant USP10 purified from insect cell lysates (final concentration 10^{-7} M) in the presence of GST–G3BP. The de-ubiquitination of HA-Ub6 was strongly inhibited by GST–G3BP (Figure 9a). Very slight inhibition was detected upon adding GST alone (Figure 9c). Moreover, GST–G3BP did not appear to inhibit USP8 (Figure 9b), once again demonstrating the specificity of the interaction between G3BP and USP10. We conclude, therefore, that G3BP negatively regulates the activity of USP10.

Discussion

In this report we describe the characterization of USP10, a new ubiquitin-specific protease. The recombinant USP10 protein produced using a baculovirus system and purified, can cleave ubiquitin chains efficiently. Similar results can be obtained when USP10 is immunoprecipitated from cell lysates. We could also detect de-ubiquitinating activity using a fragment comprising the C-terminal domain of USP10, which contains the conserved Cys and His boxes of USPs. In contrast, we were unable to detect de-ubiquitinating activity associated with the GST full-length protein expressed in bacteria, probably due to misfolding or to the need of post-translational modifications. A post-translational modification of USP10 might indeed explain why the protein runs on SDS–PAGE at approximately 100 kDa while its calculated MW is 92 900.

The biological role of USP10 is unknown at present. Using USP10 as a bait in the two-hybrid screen, we isolated G3BP, a RasGAP associated protein, recently

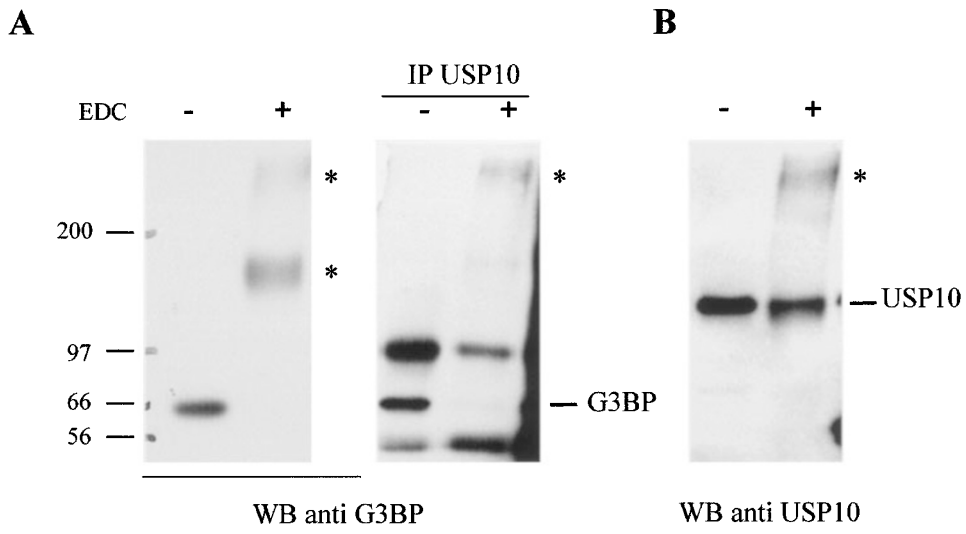

Figure 6 Chemical cross-linking analysis. U2OS total cells extract was treated with EDC and cross-linked lysate was analysed by immunoblotting with both anti G3BP (**a**) and anti USP10 (**b**) antibody. Also shown is immunoprecipitation with anti USP10 monoclonal antibody (**a**). The high molecular bands obtained by EDC treatment are labeled (*)

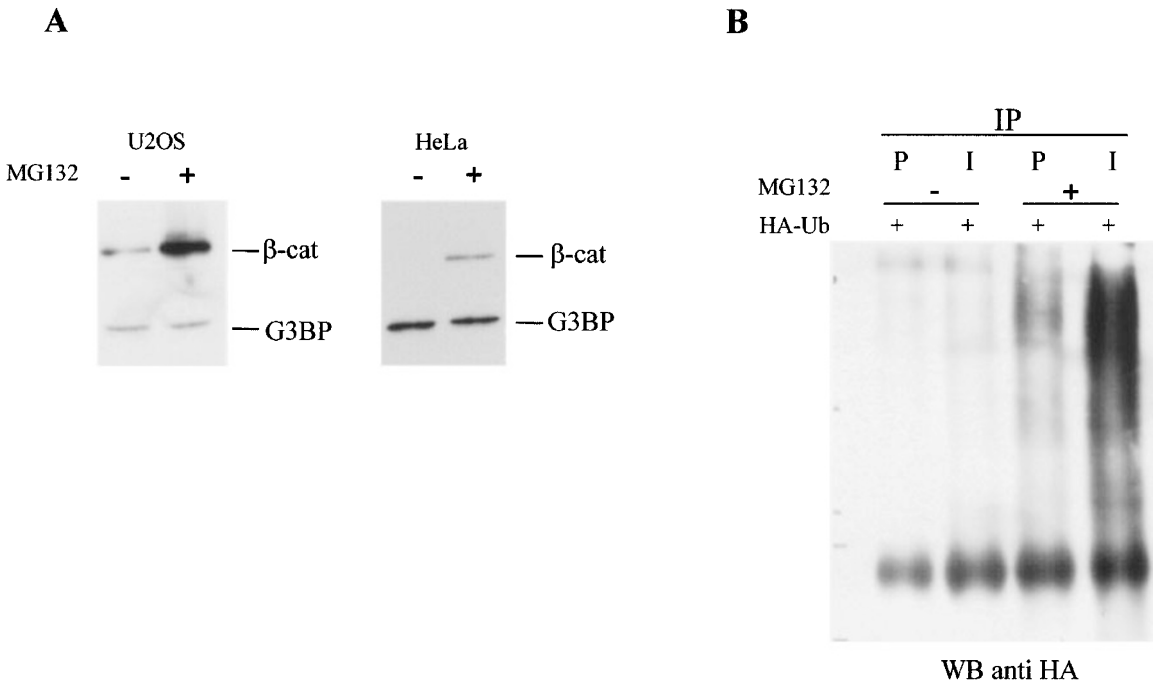

Figure 7 G3BP co-immunopurification with ubiquitinated species. (**a**) U2OS and HeLa cells were treated for 12 h with 20 μM MG132 proteasome inhibitor. G3BP protein level was assayed by Western blot analysis with anti G3BP immunopurified polyclonal antibody. β-catenin was used as treatment control. (**b**) U2OS cells were transfected with Ha-Ub6 and treated with MG132. Cell lysate was immunoprecipitated with either anti G3BP polyclonal antibody or a preimmune serum and then blotted with an anti HA monoclonal antibody

shown to be overexpressed in tumors (Guitard *et al*., 2001). We demonstrated that G3BP can bind USP10 *in vitro* and that the two proteins are associated *in vivo*.

The amino terminal region of USP10, which is not part of the domains conserved among the various USP family members, is sufficient for its interaction with G3BP. Indeed, we found that G3BP specifically interacts with USP10 and not with the other USPs tested. G3BP was first described as a protein that binds efficiently the SH3 domain of RasGAP. This interaction occurs only in proliferating cells, suggesting a recruitment of GAP–G3BP complex when Ras is in its

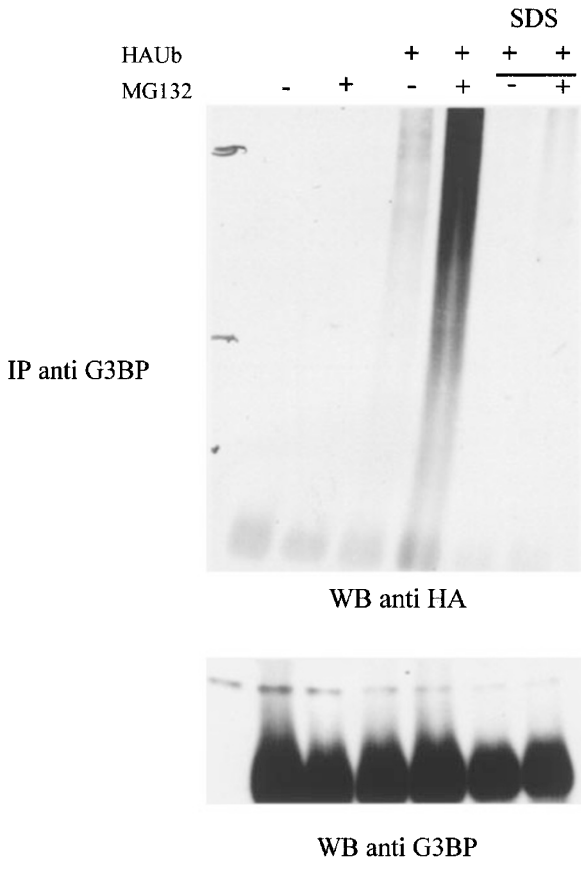

Figure 8 G3BP co-immunopurification with ubiquitinated species in denaturing buffer. U2OS cells were transfected with Ha-Ub6 and treated with MG132. Cells were lysate in NP40 or SDS buffer. Both lysates were immunoprecipitated with either anti G3BP polyclonal antibody or a preimmune serum and then blotted with an anti HA monoclonal antibody and anti G3BP polyclonal antibody

active conformation (Parker *et al*., 1996). A fraction of G3BP co-localizes with RasGAP at the plasma membrane, while much of the protein is cytosolic (Gallouzi *et al*., 1998). G3BP has two RNA binding consensus motifs RNP1 and RNP2 and an RGG box. G3BP also co-purifies with a ribonuclease activity, which is only detected when G3BP is in its hyperphosphorylated state (Gallouzi *et al*., 1998). A new DNA helicase complex was isolated which contains G3BP (Costa *et al*., 1999). More recently it was shown that G3BP is overexpressed in human tumors and that its overexpression promotes S phase entry in serum starved fibroblasts (Guitard *et al*., 2001). It was also demonstrated that this function is dependent on having an intact RNP domain.

G3BP does not appear to be a short-lived protein under the conditions examined and it does not appear to be ubiquitinated in either *in vitro* or in intact cells. It is unlikely, therefore, that G3BP itself is a substrate of USP10. In support of this Gallouzi *et al*. (1998) showed that the levels of G3BP protein are constant throughout the cell cycle. We observed that ubiquitinated bands can be detected in association with G3BP in lysates prepared from cells treated with the proteasome inhibitor MG132 (Figure 7b). These bands did not cross react with either USP10 or G3BP antibodies and are likely due to the ubiquitination of other proteins present in this complex. Since G3BP can associate with RasGAP, we tested whether RasGAP was a target for ubiquitin-dependent proteolysis. We did not detect any increase in RasGAP protein level upon addition of proteasome inhibitors. In addition we were unable to co-precipitate the G3BP complex with RasGAP, although we cannot exclude that this could be due to the fact that our antibodies recognize epitopes that are involved in such interactions.

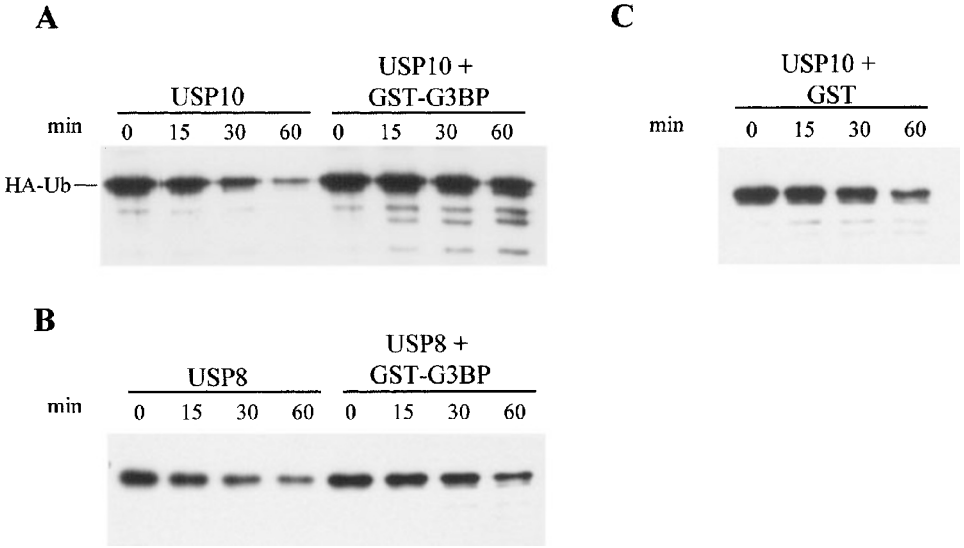

Figure 9 Effect of G3BP on USP10 activity. (**a**) Purified Baculovirus–USP10 was incubated with Ha-Ub6 in presence or absence of GSTG3BP. The reaction was follow for the time point shown and the reaction's products were analysed by Western blot with anti HA antibody. (**b**) Same as (a) but using purified baculovirus USP 8. (**c**) Same as (a) but using GST alone

Recently a mouse cDNA clone encoding a de-ubiquitinating enzyme (mUBPY) was isolated as a SH3 binding protein (Kato *et al*., 2000) and a novel SH3 domain binding site was defined. We were unable to find any SH3 binding site or domain in the USP10 sequence.

Chemical cross-linking of cell lysates followed by immunoblotting with anti USP10 and G3BP revealed a high molecular band that was immunoprecipitated by either antibody. This finding, together with the fact that by FPLC fractionation G3BP and USP10 co-purify in some high molecular weight fractions (not shown), suggest that USP10 and G3BP are part of a large macromolecular complex that contains other proteins including perhaps a USP10 substrate. We tried a classical biochemical purification of this complex by affinity chromatography using GST–USP10 against a cell lysate but we were able only to identify G3BP among the purified bands. By two-hybrid screening with G3BP as a bait, we have identified USP10 (see above) but also a number of clones that potentially represent candidate USP10 substrates.

G3BP appears to affect the activity of USP10. Upon adding G3BP to the de-ubiquitinating assay we saw an inhibition of USP10 activity. This effect was specific since G3BP did not inhibit the de-ubiquitinating activity of other USPs (Figure 9). The mechanism of inhibition should not involve competition at the active site since G3BP doesn't bind the catalytic domain of USP10.

The involvement of USPs in Ras signaling has been demonstrated. It has been reported that Ras1 interacts genetically with the Fat facets (Faf) gene during *Drosophila* eye development. Analysis of this interaction reveals that Faf has an additional function later in eye development involving Ras1 (Li *et al*., 1997). Moreover the Faf related protein Fam was shown to interact with β-catenin and AF-6, an effector of Ras, and to prevent their ubiquitination (Taya *et al*., 1998, 1999). Our finding of a physical interaction between G3BP, a RasGAP interacting protein and USP10 could provide further links between the ubiquitination pathway and Ras-mediated signaling. Future experiments will test whether the association between USP10 and G3BP (and perhaps Ras–GAP) and the inhibitory activity of G3BP on USP10 changes during the different phases of the cell cycle or upon different growth stimuli.

Materials and methods

Isolation of human USP10 and G3BP full length

USP10 partial cDNA (GenBank accession number D80012) was first identified in human myeloblast cell line KG1 by Nomura *et al*. (1994). A human cDNA library prepared from ML1 cells and cloned in Lambda Zap Express (kind gift of Kristian Helin) was screened with the first 1200 bp of USP10 partial cDNA. The probe was labeled with ^{32}PdCTP by random primer method (Amersham), and hybridization was performed in: 5× Denhard's solution, 0.5% SDS, 5× SCC and 100 μg/ml salmon sperm DNA for 16 h at 65°C. A total of 1×10^6 recombinant phages were screened and 80 positives were found. Sixteen positive plaques were identified from the secondary screening. These plaques were screened then by PCR using *pfu* DNA polymerase (Stratagene) and the primers utilized are: T3 universal primer as the upstream primer, and an internal USP10 primer with the following sequence: 5′-CGAGGAGTCACAAAAGAATTG-3′ as downstream primer. The resulting PCR products were cloned directly into pCR 2.1 vector (Invitrogen) and screened for inserts by restriction digestion. Clones containing the largest inserts were selected for sequencing. These clones provided the 5′ region of USP10 cDNA were cloned into pBSKKIA190 by insertion at the *Hinc*II and *Eco*RI sites to generate a putative full-length.

Human G3BP cDNA was obtained by PCR screening of a cDNA library made from human lung embryo fibroblast (kind gift of Pier Paolo Di Fiore). The reaction was performed using *pfu* DNA polymerase (Stratagene) and the following primers, designed on the human G3BP cDNA sequence (GenBank accession number U32519): 5′-GAC-CAAAGCAATGGTGATGG-3′ as upstream primer and 5′-CATGAAGATTACTGCCGTGG-3′ as downstream primer. The resulting PCR products were cloned directly into pCR 2.1 vector (Invitrogen) and screened for inserts by restriction digestion. The accuracy of the cDNA was confirmed by sequencing.

Plasmids

The Cys(488)Ala mutation was introduced into USP10 by a two step PCR-based approach. The 5′-GGAACTGGGCC-TACATTAATGC-3′ and 5′-GCATTAATGTAGGCC-CAGTTCC-3′ primers were used to mutate the Cys 488 codon together with the following flanking primers: 5′-TTGTTCCGGTTTCAGAGGATCC-3′, upstream the Cys codon at the *Bam*HI site and 5′-CAGACAGGCTT-GACTTGTTAAC-3′, downstream the Cys codon at the *Hpa*I site. The final PCR product was used to replace the *Bam*HI–*Hpa*I wild-type fragment in the pSKUSP10 construct. The mutant was verified by DNA sequencing.

pGEXUSP10 was constructed by cloning the *Eco*RI–*Not*I USP10 fragment into pGEX4–T1 expression vector (Pharmacia Biotech), while pGEXG3BP was obtained cloning G3BP *Eco*RI fragment into the same expression vector. Eukaryotic expression vector pBMN–GFP was used to overexpress wild-type and mutant USP10. The constructs were obtained cloning the *Xho*–*Not*I pSKUSP10 wild-type or mutant fragment in the same sites of the vector.

Yeast two-hybrid screens

A bait was made as a fusion between LexA DNA binding domain (amino acids 1–211) and USP10 (amino acids 14–862). The second hybrid is a fusion between a nuclear localized VP16 acidic activation domain and random cDNA fragments derived from 9.5 and 10.5 day-old mouse embryos (kind gift of Isabel Chiu). These two hybrids are co-expressed in the *S. cerevisiae* strain L40 that contains two integrated reporter constructs, the yeast HIS3 gene and the bacterial lacZ gene, which contain binding sites for the LexA protein. Approximately 10^8 yeast transforms were screened, 30 clones were found positive on Xgal/-Hist plates in the presence of 5 mM 3-aminotriazole. The recovered plasmids were subjected to restriction and sequence analysis and from this

screen 10 clones were identified that correspond to the same cDNA. Using the same system we screened a human lymphocyte cDNA library (Clontech) and we isolated 12 positives, one of which turned out to be the human homologous of one of the mouse clones.

In vitro *binding assay* GST and GSTUSP10 were produced using standard methods. Briefly, protein expression was induced by addition of 0.1 mM IPTG to a bacteria culture at OD_{600} 0.6. The bacteria were lysed after 4 h of growth at 37°C, in 1% Triton in PBS plus 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin.

After sonication and centrifugation, the supernatant was incubated with glutathione Sepharose beads (Pharmacia) rotating for 1 h at 4°C. After three washes in ice-cold PBS, bound proteins were eluted with 10 mM glutathione in 50 mM Tris HCl pH 8.0. Full-length human G3BP was *in vitro* translated using TNT reticulocyte lysate (Promega) following the instructions of the manufacturer. Binding reactions were performed in 600 μl of 20 mM HEPES pH 7.4, 150 mM NaCl, 0.05% NP40, 10% glyerol; for 1 h at 4°C, using from 5 to 20 μg of GST fusion proteins and 15 μl of the *in vitro* translated product. Glutathione Sepharose was added (30 μl from a 50% slurry) and the reactions continued for 30 min with rotation. The beads were pelleted, washed three times in the binding buffer, resuspended in 2× Laemmli sample buffer and heated at 95°C for 10 min. After separation on a SDS–PAGE the gel was dried and exposed.

Immunoprecipitation and immunoblotting

Protein extracts was prepared from human U2OS, HeLa or IMR90 cell line by scraping the cells directly in lysis buffer: 50 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton, 5 mM EGTA, 1.5 mM $MgCl_2$, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 0.5 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin. Lysates were incubated on ice for 20 min and then centrifuged at 14 000 r.p.m. for 15 min at 4°C. Supernatants were assayed for total protein using the Bradford method (BioRad) according to the instructions provided. For immunoprecipitation: typically 1 mg of cells lysate was diluted to 500 μl with NET buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% Triton, 1 mM EDTA) and incubated on ice for 60 min, in the presence of 2 μl of rabbit polyclonal antiserum or 200 μl of monoclonal supernatant. Fifty μl of 50% slurry of Protein A-Sepharose (Pharmacia) were added and the reactions incubated for 30 min at 4°C on a gentle-rotating mixer. Pellets were washed three times with NET buffer resuspended in 20 μl of 2× Laemmli sample buffer and heated at 95°C for 10 min prior to loading on a 8% SDS–PAGE. Immunoblotting was performed according to standard methods. The ECL-enhanced chemiluminescence kit (Amersham) was used for detection.

Antiserum

Polyclonal anti-USP10 and anti-G3BP antiserums were generated by PRIMM (Milan, Italy). Rabbits were injected with purified GSTUSP10 or GSTG3BP fusion proteins. Monoclonal antibody were raised using the same antigens. Mouse anti ubiquitin antibody was purchased from ZYMED Laboratories Inc. and monoclonal anti HA antibody from BabCO.

Chemical cross-linking

Proliferating human U2OS or IMR90 cells were collected, lysed and treated with 10 mM 1-ethyl-3(dimethylamino)-propyl carbodimide (EDC) as described (Gallouzi *et al*., 1998) with minor modification.

De-ubiquitinating assay

The assay mixtures (50 μl) contained reaction buffer (50 mM Tris HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT), and recombinant purified USP10 or USP 8 (kind gift of Dr Jorge Dominguez) from insect cells infected with baculovirus expressing His tag USP10 or USP 8 (from 10 up to 100 nM), was incubated from 0 to 2 h at 37°C, with 0.18 μg of recombinant Ha-ubiquitin six-mer (Ub6). Ten μl were taken each time point and the reaction was stopped adding 10 μl of 2× Laemmli sample buffer and heated at 95°C for 5 min. The products were analysed by 10% SDS–PAGE, and blotted against anti-HA purified MBA. Five mM N-ethylmaleimide (NEM) was used as UBPs chemical inhibitor. The cDNA encoding HA-tagged ubiquitin was excise from the parental plasmid pMT123 (Treier *et al*., 1994) and insert into pET23 bacterial expression vector to transform BL21 bacteria. Purification of the recombinant HAUb6 from bacterial supernatant was performed onto S200 16/60 column, the cleanest fractions were pooled, concentrated and used as substrate for USP10 de-ubiquitinating activity. To test the effect of G3BP on USP10 activity 5 molar excess of GST or GSTG3BP was added to the reactions. The assay was also performed with immunoprecipitated USP10 from human cells.

Purification of recombinant USP10 from insect cells infected with baculovirus expressing His tag USP10. Bac-to-Bac® Baculovirus Expression Systems (Life Technologies) was used to obtain the recombinant proteins. Wild-type and mutant USP10 were cloned into pFastBac HT expression vector (Life Technologies) contain 6x-histidine tag. Monolayer cultures of Sf9 were infected with pFastBac HT-USP10 wt or mutant. The proteins were purified according to the instructions provided with minor modification using Pro-Bound™ Resin (Invitrogen) as column matrix.

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