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UBPY: a growth-regulated human ubiquitin isopeptidase

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The ubiquitin pathway has been implicated in the regulation of the abundance of proteins that control cell growth and proliferation. We have identified and characterized a novel human ubiquitin isopeptidase, UBPY, which both as a recombinant protein and upon immunoprecipitation from cell extracts is able to cleave linear or isopeptide-linked ubiquitin chains. UBPY accumulates upon growth stimulation of starved human fibroblasts, and its levels decrease in response to growth arrest induced by cell–cell contact. Inhibition of UBPY accumulation by antisense plasmid microinjection prevents fibroblasts from entering S-phase in response to serum stimulation. By increasing or decreasing the cellular abundance of UBPY or by overexpressing a catalytic site mutant, we detect substantial changes in the total pattern of protein ubiquitination, which correlate stringently with cell proliferation. Our results suggest that UBPY plays a role in regulating the overall function of the ubiquitin–proteasome pathway. Affecting the function of a specific UBP *in vivo* could provide novel tools for controlling mammalian cell proliferation.

Keywords: cell proliferation/growth/ubiquitin isopeptidase/UBPY

Introduction

The selective degradation of proteins through the ubiquitin–proteasome pathway involves the activation of a signaling cascade that generates the covalent attachment of a polyubiquitin chain to protein targets (Hochstrasser, 1995). This modification occurs through the formation of isopeptide bonds between the C-terminus of ubiquitin and the ε-amino groups of lysine residues on the target protein. A polyubiquitin chain formed through the addition of multiple ubiquitin molecules to the target acts as a signal for degradation by the proteasome, a large (26S) multimeric protein complex (Goldberg, 1995). Ubiquitin conjugation requires the presence of three key enzymes, E1, E2 and E3. In the presence of ATP, the E1 or ubiquitin-activating enzyme binds ubiquitin, forming a thioester bond between a cysteine in its active site and the C-terminus of a glycine residue of ubiquitin. Ubiquitin is then transferred to an E2 (ubiquitin-conjugating enzyme, UBC) which can either directly transfer ubiquitin to a lysine residue on the target substrate, or transfer its ubiquitin to an E3 enzyme (ubiquitin ligase). E3s appear specifically to recognize substrates and catalyze the formation of ubiquitin isopeptides. The multiplicity of E2 and E3 enzymes is thought to be responsible for the specific targeting of protein substrates for degradation (Kumar *et al*., 1997).

De-ubiquitinating activities can promote the accumulation of ubiquitin in a given cell by cleaving linear polymers of ubiquitin as they emerge from ribosomes, or ubiquitin–isopeptide trees which are formed after polyubiquitinated proteins are cleaved by the proteasome (Ciechanover, 1994; Wilkinson, 1997). De-ubiquitinating enzymes are also thought to counteract the effects of E2/E3-mediated conjugation by removing the polyubiquitin chain from conjugated proteins prior to their degradation by the proteasome (Kalderon, 1996; Rolfe *et al*., 1997). This might either represent a mean of preventing degradation by the proteasome, or might be part of those ubiquitination processes not aimed at directing protein degradation. De-ubiquitinating enzymes can be subdivided into two broad groups: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin isopeptidases (UBPs) (Wilkinson, 1997). As far as UBPs are concerned, although direct evidence of their substrate specificity and their biochemical regulation *in vivo* is lacking at present, a number of published reports indicate that certain UBPs have highly specific functions. Indeed, genes encoding UBPs have been isolated in different organisms because of the genetic defects caused by their inactivation (Baker *et al*., 1992; Fischer-Vize *et al*., 1992; Papa and Hochstrasser, 1993; Henchoz *et al*., 1996; Kalderon, 1996; Moazed and Johnson, 1996). As for their biochemical characterization, a member of this family, isopeptidase T (IsoT), has been studied in great detail (Falquet *et al*., 1995a,b; Wilkinson *et al*., 1995). The enzyme is able to cleave both linear and isopeptide-linked ubiquitin, and it appears to require a free ubiquitin C-terminus for optimal activity (Wilkinson *et al*., 1995).

Given the number and relevance of key cell cycle and proliferation regulators whose levels are regulated by ubiquitination (Pagano *et al*., 1995a; Draetta and Pagano, 1996; Pagano, 1997), we became interested in the identification of human UBPs which play a role in growth and cell cycle control. A number of mammalian UBPs appear to play a role in growth regulation (Huebner *et al*., 1988; Nakamura *et al*., 1988, 1992; Papa and Hochstrasser, 1993; Gupta *et al*., 1994; Falquet *et al*., 1995a,b; Gray *et al*., 1995; Wilkinson *et al*., 1995; Zhu *et al*., 1996, 1997; Everett *et al*., 1997; Jaster *et al*., 1997). Here we have characterized UBPY, a novel ubiquitin isopeptidase.

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        10         20         30         40         50         60         70
MPAVASVPKE LYLSSSLKDL NKKTEVKPEK ISTKSYVHSA LKIFKTAEEC RLDRDEERAY VLYMKYVTVY

        80         90        100        110        120        130        140
NLIKKRPDFK QQQDYFHSIL GPGNIKKAVE EAERLSESLK LRYEEAEVRK KLEEKDRQEE AQRLQQKRQE

       150        160        170        180        190        200        210
TGREDGGTLA KGSLENVLDS KDKTQKSNGE KNEKCETKEK GAITAKELYT MMTDKNISLI IMDARRMQDY

       220        230        240        250        260        270        280
QDSCILHSLS VPEEAISPGV TASWIEAHLP DDSKDTWKKR GNVEYVVLLD WFSSAKDLQI GTTLRSLKDA

       290        300        310        320        330        340        350
LFKWESKTVL RNEPLVLEGG YENWLLCYPQ YTTNAKVTPP PRRQNEEVSI SLDFTYPSLE ESIPSKPAAQ

       360        370        380        390        400        410        420
TPPASIEVDE NIELISGQNE RMGPLNISTP VEPVAASKSD VSPIIQPVPS IKNVPQIDRT KKPAVKLPEE

       430        440        450        460        470        480        490
HRIKSESTNH EQQSPQSGKV IPDRSTKPVV FSPTLMLTDE EKARIHAETA LLMEKNKQEK ELRERQQEEQ

       500        510        520        530        540        550        560
KEKLRKEEQE QKAKKKQEAE ENEITEKQQK AKEEMEKKES EQAKKEDKET SAKRGKEITG VKRQSKSEHE

       570        580        590        600        610        620        630
TSDAKKSVED RGKRCPTPEI QKKSTGDVPH TSVTGDSGSG KPFKIKGQPE SGILRTGTFR EDTDDTERNK

       640        650        660        670        680        690        700
AQREPLTRAR SEEMGRIVPG LPSGWAKFLD PITGTFRYYH SPTNTVHMYP PEMAPSSAPP STPPTHKAKP

       710        720        730        740        750        760        770
QIPAERDREP SKLKRSYSSP DITQAIQEEE KRKPTVTPTV NRENKPTCYP KAEISRLSAS QIRNLNPVFG

       780        790        800        810        820        830        840
GSGPALTGLR NLGNTCYMNS TLQCLCNAPH LADYFNRNCY QDDINRSNLL GHKGEVAEEF GIIMKALWTG

       850        860        870        880        890        900        910
QYRYISPKDF KITIGKINDQ FAGYSQQDSQ ELLLFLMDGL HEDLNKADNR KRYKEENNDH LDDFKAAEHA

       920        930        940        950        960        970        980
WQKHKQLNES IIVALFQGQF KSTVQCLTCH KKSRTFEAFM YLSLPLASTS KCTLQDCLRL FSKEEKLTDN

       990       1000       1010       1020       1030       1040       1050
NRFYCSHCRA RRDSLKKIEI WKLPPVLLVH LKRFSYDGRW KQKLQTSVDF PLENLDLSQY VIGPKNNLKK

      1060       1070       1080       1090       1100       1110       1120
YNLFSVSNHY GGLDGGHYTA YCKNAARQRW FKFDDHEVSD ISVSSVKSSA AYILFYTSLG PRVTDVAT..
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Fig. 1. Amino acid sequence of human UBPY. The predicted amino acid sequence of the UBPY cDNA (DDBJ/EMBL/GenBank accession No. P40818) is depicted. The sequence between amino acids 9 and 188, used to generate UBPY antibodies, is underlined. The cysteine and histidine boxes, hallmarks of the UBP family (Papa and Hochstrasser, 1993; Wilkinson *et al*., 1995) are highlighted.

UBPY is a growth-regulated UBP which appears to play a critical role in controlling the overall function of the ubiquitin–proteasome pathway.

Results

UBPY characterization

UBPY is the predicted product of a human cDNA identified in human myeloblasts (Nomura *et al*., 1994). The sequence of the UBPY open reading frame (ORF) is shown in Figure 1 and it displays the typical hallmarks of the UBP family of de-ubiquitinating enzymes, including the so called histidine and cysteine boxes (Wilkinson *et al*., 1995; Wilkinson, 1997) (highlighted in Figure 1).

A cDNA fragment encoding amino acids 9–188 of UBPY, whose sequence does not show significant similarities to other known UBPs (underlined region in Figure 1), was inserted into a pGEX expression vector and used to generate a GST fusion protein in bacteria. The expressed GST–UBPY 9–188 protein was purified through chromatography on glutathione–Sepharose and used to generate rabbit polyclonal antisera. The obtained sera were affinity purified on Amino-Link-conjugated GST–UBPY 9–188 after removal of the anti-GST component by affinity chromatography onto immobilized GST. In Figure 2 we present the characterization of the UBPY protein in a panel of human cell lines. A protein doublet of M_r 130 kDa was recognized specifically upon immunoblotting with anti-UBPY antibodies in lysates from both non-transformed human fibroblasts (strain WI-38) and the U2OS human osteosarcoma cell line (Figure 2A). The antibodies were able to precipitate the protein from cell

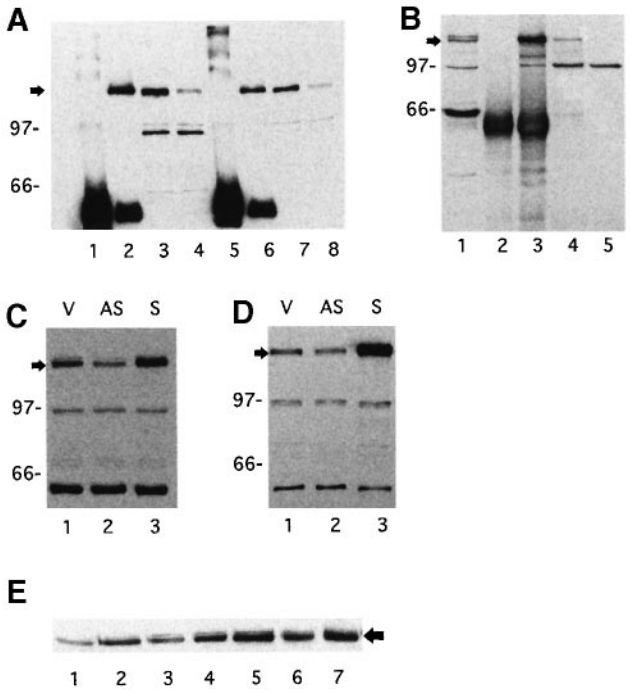

Fig. 2. Identification of the UBPY protein in cell extracts. (A and B) Immunoprecipitation and immunoblotting experiments. (**A**) U2OS (lanes 1–4) and WI-38 (lanes 5–8) cell extracts were prepared. An aliquot (1 mg) of lysate proteins was immunoprecipitated with either affinity-purified UBPY antibodies (lanes 2 and 6) or the corresponding pre-immune serum (lanes 1 and 5). Thirty μg of cell lysate (lanes 3 and 7) or of an anti-UBPY immunoprecipitation supernatant (lanes 4 and 8) were subjected to SDS–PAGE (8%), transferred to nitrocellulose and blotted with the UBPY immune serum. (**B**) The UBPY cDNA was transcribed and translated in RRL. Five μl of the programed RRL (lane 4) or of the control (empty vector) mixture (lane 5), one half of an immunoprecipitation (500 μg of C33A lysate) with immune serum (lane 3) or pre-immune serum (lane 2) and 10 μg of lysate (lane 1), were subjected to SDS–PAGE (10%) and processed as in (A). (C and D) UBPY immunoblotting analysis upon transient transfection. (**C**) C33A and (**D**) U2OS cells were transfected with the pCMV-neo-Bam control vector (lane 1) or with the same vector containing the UBPY cDNA in either sense (S) (lane 3) or antisense (AS) orientation (lane 2). Twenty μg of each lysate were subjected to SDS–PAGE (8%) and processed as in (A). (**E**) Immunoblotting from human cell lines. Twenty μg of total lysate from the indicated cell lines were subjected to anti-UBPY immunoblotting as in (A). Lane 1, SAOS; 2, U2OS; 3, WI-38; 4, VA13; 5, C33A; 6, HeLa; 7, U937. Molecular size markers (kDa) are indicated. The position of UBPY is indicated by arrowheads.

lysates and to deplete them quantitatively of UBPY (Figure 2A). A similar experiment performed in C33A cervical carcinoma cells shows that the lower band of the UBPY doublet co-migrated with *in vitro* translated UBPY (Figure 2B).

To confirm the identity of the protein doublet, we performed transfection experiments using both U2OS and C33A cells. We generated a cDNA construct containing the UBPY ORF under the transcriptional control of a cytomegalovirus (CMV) promoter in sense or antisense orientation. Forty-eight hours after transfection, cells were collected and lysates analyzed by immunoblotting with anti-UBPY antiserum. In lysates from cells transfected with the antisense vector, we detected a decrease in the levels of both 130 kDa bands as compared with lysates made from cells transfected with vector alone (Figure 2C and D). Conversely, lysates from cells transfected with

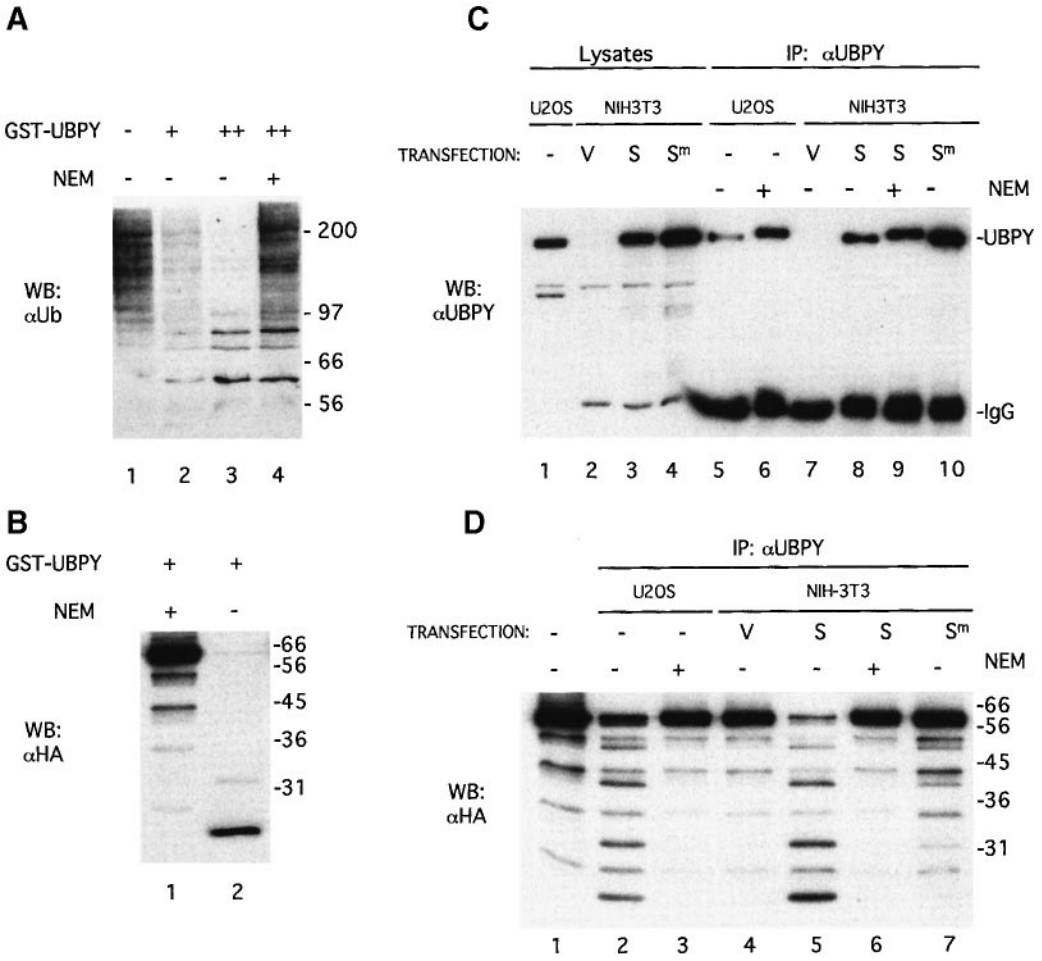

Fig. 3. UBPY de-ubiquitinating activity. (A and B) GST–UBPY protein was expressed in bacteria, purified and its de-ubiquitinating activity tested. (**A**) *In vitro* ubiquitinated and NEM-inactivated RRL (Promega) was used as a substrate and incubated in the absence (lane 1) or presence of 0.5 (lane 2, indicated as +) or 1 μg of GST–UBPY (lane 3, indicated as ++), or of 1 μg of GST–UBPY pre-treated with NEM (lane 4). Reactions were stopped with Laemmli buffer and subjected to immunoblotting with anti-ubiquitin antiserum (Sigma). (**B**) Ub6 (see Materials and methods) was used as a substrate and incubated in the presence of 1 μg of GST–UBPY (lane 2) or 1 μg of GST–UBPY pre-treated with NEM (lane 1). Reactions were stopped with Laemmli buffer and subjected to immunoblotting with monoclonal anti-HA antibodies. (C and D) Testing UBPY activity in immunoprecipitations. (**C**) Cell extracts from U2OS wild-type (lanes 1, 5 and 6) and from NIH 3T3 cells transiently transfected with pCMV-neo-Bam vector alone (lanes 2 and 7), or with the same vector containing the UBPY cDNA coding for UBPY wild-type (lanes 3, 8 and 9) or UBPY Cys786Ala mutant proteins (lanes 4 and 10), were prepared, and immunoprecipitations from 300 μg of each cell extract with the affinity-purified UBPY antibodies (lanes 5–10) were performed in the presence (lanes 6 and 9) or absence (lanes 5, 7 and 10) of 10 mM NEM. Forty μg of cell extracts (lanes 1–4) and one half of the total immunoprecipitation (lanes 5–10) were subjected to anti-UBPY immunoblotting. (**D**) Ub6 was used as a substrate and incubated solely with protein A–Sepharose beads (lane 1) or with UBPY immunoprecipitations (lanes 2–7). Reactions were stopped with Laemmli buffer and subjected to immunoblotting with anti-HA monoclonal antibody to detect HA-tagged reaction products. Lanes 2–7 measure the activities of the samples indicated in (C) (lanes 5–10 above).

sense UBPY vector showed a substantial accumulation of both bands compared with control. It should be pointed out that the data presented are an underestimate of the actual effects of sense/antisense transfections since the transfection efficiency was ~30%, while the immunoblots reflected protein expression in the total cell population. We can therefore estimate a nearly quantitative depletion of UBPY protein in cells transfected with antisense plasmid and, conversely, a substantial increase in UBPY levels in sense-transfected cells. Our data demonstrate that the anti-UBPY antibodies recognize a protein of the expected size in human cell lysates. The UBPY doublet, which in some cases even appeared as a triplet, could result from a post-translational modification, and it was detected in all human cell lines tested (Figure 2E). Indeed, we have evidence that UBPY is a phosphoprotein (C.Mercurio, S.Naviglio and G.F.Draetta, unpublished).

UBPY enzymatic activity

To demonstrate that UBPY is a UBP, we generated a pool of ubiquitinated proteins using rabbit reticulocyte lysate (RRL) and then incubated them with purified full-length recombinant GST–UBPY (see Materials and methods). To block endogenous de-ubiquitinating or ubiquitin-conjugating activities, prior to the addition of GST–UBPY the RRL was treated with 20 mM *N*-ethylmaleimide (NEM) and then the NEM was removed by centrifugation and washing through Centricon filters (see Materials and methods). In Figure 3A we show the result of an experiment in which after the incubation with or without GST–UBPY, samples were analyzed by SDS–PAGE and immunoblotted with anti-ubiquitin antibodies. Incubation with UBPY resulted in the disappearance of most of the ubiquitin immunoreactivity (the bands detected at 60–90 kDa in the presence of UBPY were caused by

antibody cross-reactivity with the GST–UBPY preparation). Under the same conditions, a GST–UBPY protein treated with NEM prior to incubation with the ubiquitinated substrates failed to generate de-ubiquitination. The disappearance of ubiquitinated bands from the blot was not paralleled by a disappearance of proteins as detected by Ponceau red staining, demonstrating that the UBPY preparation was free of contaminating proteolytic activities; furthermore, no effect was seen upon incubation of the sole GST moiety with the RRL proteins (data not shown). The above results show that UBPY can hydrolyze ubiquitin–isopeptide bonds.

UBPY could also cleave purified linear ubiquitin chains. In Figure 3B, the results of an experiment in which GST–UBPY was incubated with hemagglutinin (HA)-tagged ubiquitin hexamer (Ub6, see Materials and methods) are shown. Furthermore, as is the case for other UBPs, UBPY was able to cleave ubiquitin–β-galactosidase fusion proteins upon expression in *Escherichia coli* (data not shown).

To study the biological role of human isopeptidases, it is essential to be able to measure the de-ubiquitinating activity of UBPs directly isolated from cell extracts rather than from recombinant sources. To achieve this, we set up an assay to measure the de-ubiquitinating activity in anti-UBPY immunoprecipitations, by testing the cleavage of Ub6. We employed lysates from either U2OS cells which express UBPY or from NIH 3T3 cells in which our antibodies do not detect UBPY protein. NIH 3T3 cells were transfected with various UBPY constructs. Upon transfection with a sense cDNA construct, NIH 3T3 cells expressed the protein to amounts comparable with the endogenous levels detected in U2OS cells (Figure 3C). Both a wild-type protein and a Cys786Ala mutant could be expressed in NIH 3T3 cells and immunoprecipitated using UBPY antibodies. In Figure 3D, we show that a detectable NEM-sensitive de-ubiquitinating activity could be revealed in immunoprecipitations from U2OS cell extracts and that immunoprecipitations from NIH 3T3 cells transfected with wild-type, but not a Cys786 mutant UBPY construct, expressed a detectable NEM-sensitive de-ubiquitinating activity. These experiments demonstrate that: (i) endogenous UBPY is an active de-ubiquitinating enzyme; (ii) the UBPY expressed upon transfection is also active; and (iii) as predicted, a mutation of Cys786 inactivates the enzyme. These results also imply that by up-regulating UBPY levels, an increase in its cellular enzymatic activity can be generated (see also below).

UBPY regulation

We next studied the regulation of UBPY levels in response to changes in cell growth conditions. We analyzed the expression of the protein upon removal of growth factors. WI-38 human fibroblasts were deprived of serum for 72 h to induce G_0 entry and then re-stimulated by the addition of medium containing 10% fetal bovine serum. At different times after serum stimulation, samples were analyzed for cell cycle status by propidium iodide flow-cytometry and for UBPY and cell cycle marker expression by immunoblotting. UBPY expression was undetectable in serum-deprived cells and reappeared as cells progressed through the G_1 phase of the cycle (Figure 4A and B).

Based on the above results, we asked whether the up-

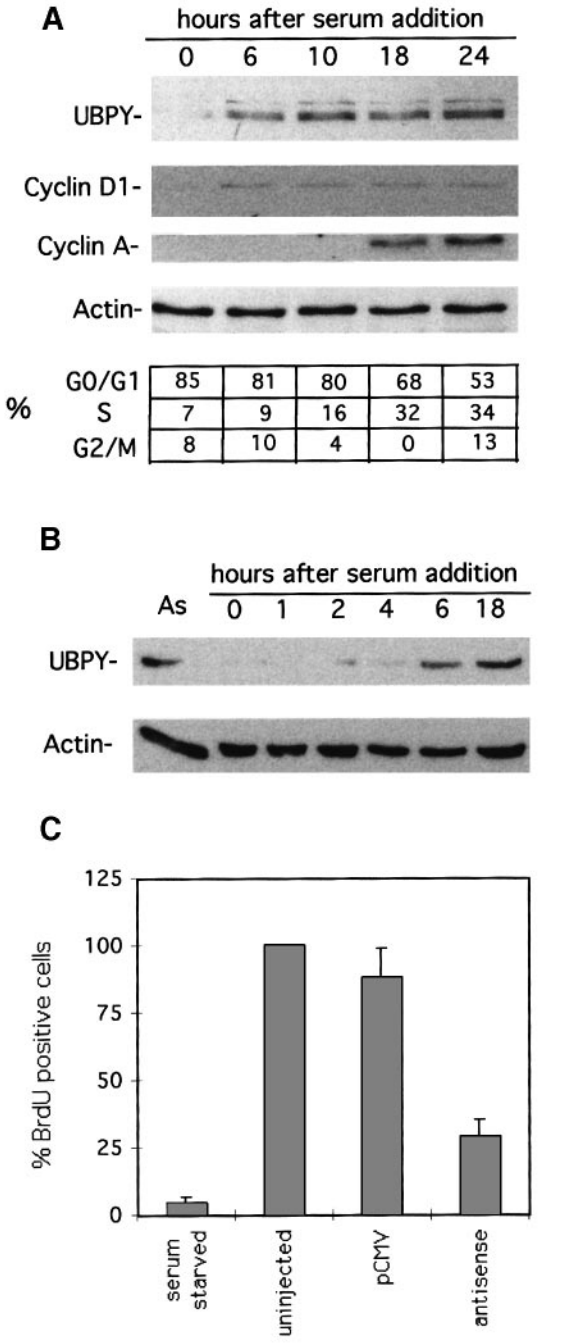

Fig. 4. UBPY plays a critical role in cell growth. (**A** and **B**) Regulation of UBPY protein levels in response to changes in growth conditions. WI-38 human fibroblasts were arrested in G_0/G_1 phase by serum starvation for 72 h (0.5% serum), re-stimulated with 10% serum and cultured further for the indicated times. Cell extracts were prepared from exponentially (As) growing, starved and re-stimulated cells. Twenty μg of extracts were subjected to SDS–PAGE (8%), transferred to nitrocellulose membranes and blotted with antibodies against the indicated proteins. The different cell cycle phases were determined by propidium iodide flow-cytometry. (**C**) Microinjection of antisense UBPY cDNA causes inhibition of S-phase entry. WI-38 human fibroblasts were made quiescent by serum starvation. Arrested cells (200–250 per point) were injected with the pCMV-neo-Bam empty vector or the same vector containing the UBPY cDNA in the antisense orientation. Cells were re-stimulated with 10% serum and BrdU was added to the growth medium. Cells were fixed and stained 24 h post-serum addition. The indicated values were calculated as the ratio of injected BrdU-positive cells to BrdU-positive surrounding cells multiplied by 100. The results are the mean ± SE of three independent experiments.

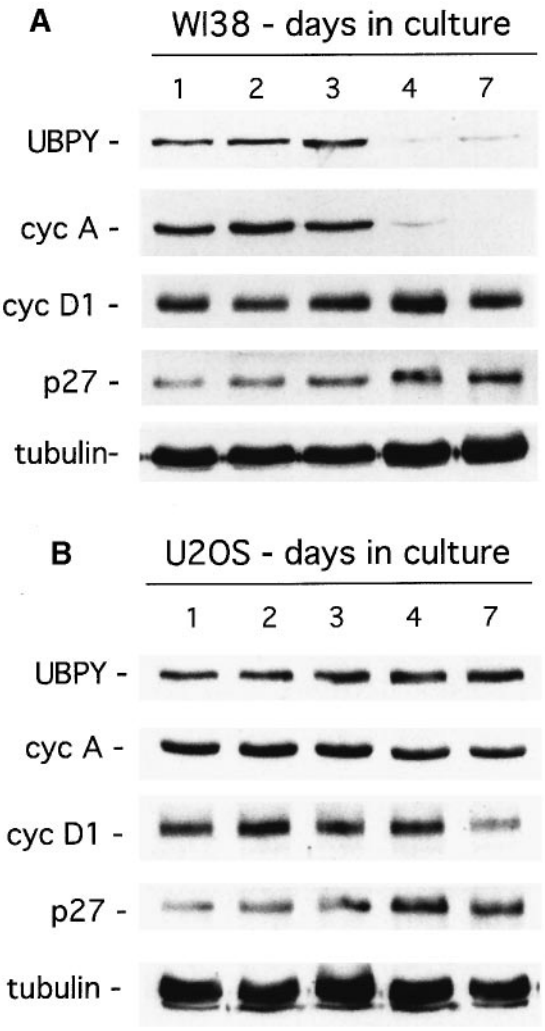

Fig. 5. Regulation of UBPY protein levels by cell–cell adhesion. (**A**) WI-38 human fibroblasts (8×10^5 cells/plate) and (**B**) human osteosarcoma U2OS cells (5×10^5 cells/plate) were plated and cultured until they reached confluence (day 4) and were then grown for a further 3 days (day 7), with medium changes every 2 days. Cell extracts were prepared from asynchronously growing (days 1–3) and confluent cells (days 4 and 7). Twenty μg of each lysate were subjected to SDS–PAGE (12%), transferred to nitrocellulose membrane and blotted with antibodies against the indicated proteins.

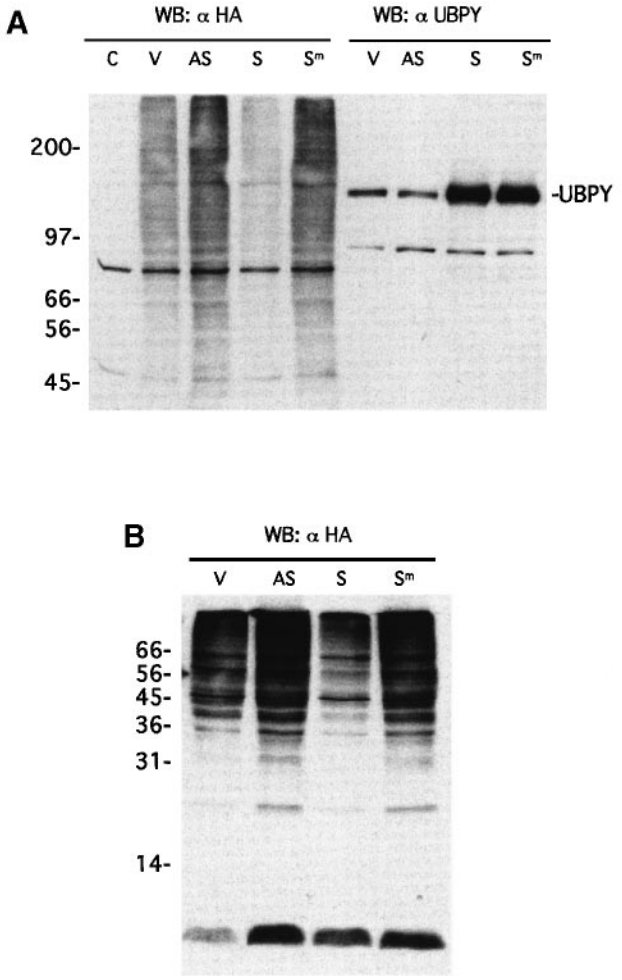

Fig. 6. Effects of altering UBPY expression on protein ubiquitination. U2OS cells were co-transfected with 10 μg of HA-Ub plasmid (see Materials and methods) and 10 μg of pCMV empty vector (V), PCMV–UBPY antisense (AS), PCMV–UBPY sense (S) or PCMV–UBPY Cys786Ala sense (S^m) constructs. (**A**) Lysates (30 μg) were subjected to SDS–PAGE (8%) and to anti-UBPY or anti-HA immunoblotting. A control transfection (lane C) with 20 μg of pCMV empty vector without HA-Ub plasmid was also performed. (**B**) Lysates (30 μg) from a different experiment were subjected to SDS–PAGE (15%) and to anti-HA immunoblotting to detect ubiquitinated proteins. In both the experiments, each co-transfection was made in triplicate.

regulation of UBPY levels observed in response to serum stimulation was an event necessary for cell growth. To answer this question, we employed microinjection of UBPY antisense cDNA constructs and measured the effects of preventing its appearance on cell cycle re-entry. Figure 4C shows that in comparison with controls, in three separate experiments, cells microinjected with antisense constructs failed to enter S-phase as shown by the reduced incorporation of bromodeoxyuridine (BrdU) added together with serum for 24 h.

We also maintained cells in culture until they had reached confluence (Figure 5). In non-immortalized human fibroblasts, which are inhibited by cell–cell contact, we observed a down-regulation of the protein coincident with the cells becoming arrested, as monitored by the disappearance of cyclin A and the accumulation of the p27 Cdk-inhibitory protein (Guadagno *et al*., 1993; Sherr and Roberts, 1995) (Figure 5A). Interestingly, in the transformed osteosarcoma line U2OS, which does not show contact inhibition, such down-regulation of the UBPY protein levels was not observed, despite the fact that cells became confluent as monitored by the increase in p27 levels (Figure 5B). Similar results were obtained in other transformed cell lines, including normal fibroblasts made to express SV40 large T antigen (data not shown). Altogether, the above experiments identify UBPY as a growth-regulated protein that plays a critical role in cell proliferation.

Effects of altering UBPY expression on protein ubiquitination and cell growth

To identify biochemical changes related to altered UBPY expression, we analyzed the overall protein ubiquitination status in extracts from cells transiently transfected with various UBPY constructs along with HA-Ub plasmids (see Materials and methods; Figure 6). It has been demon-

strated that 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). The HA-Ub allowed the quantitative detection of protein ubiquitination by immunoblotting. To our surprise, we consistently found that by either inhibiting UBPY accumulation with an antisense construct (AS in Figure 6), or by inducing the expression of a Cys786 mutant of the protein (S^m in Figure 6), a substantial increase in total cell protein ubiquitination occurred. Conversely, upon overexpression of the wild-type protein (S in Figure 6), a decrease in protein ubiquitination was found. The results of a separate experiment (Figure 6B) in which samples were run on a higher density gel show that even lower molecular weight ubiquitinated species accumulated in antisense or mutant transfected cells (AS or S^m). The observed biochemical changes were not generated *in vitro* since NEM was added at the time of cell lysis. These experiments indicate that UBPY is essential for the execution of a key general step in ubiquitin processing.

On the basis of the results obtained with the transfection experiments, we decided to evaluate the biological consequences of altering UBPY function. We show above (Figure 4C) that UBPY antisense microinjection in starved fibroblasts, which are depleted of the protein, results in an inhibition of S-phase entry. In U2OS cells also, altering UBPY expression caused dramatic changes. Examples of our findings are shown in Figure 7. Down-regulation of UBPY levels in asynchronously growing U2OS cells by antisense plasmid transfection determined an accumulation of cells in the S-phase of the cell cycle. To measure this, we used a modification of the technique described by van den Heuvel and Harlow (1993) which makes use of a marker gating flow-cytometry procedure to identify transfected cells. Using co-transfection of the UBPY antisense plasmid and of a plasmid encoding a modified green fluorescent protein (GFP; see Materials and methods), we were able to assess changes in cell cycle distribution resulting from UBPY down-regulation. By increasing the dosage of UBPY antisense plasmid, we could observe a progressive increase in S-phase cell accumulation (Figure 7A). The magnitude of these effects is similar to, if not greater than, the results obtained upon acute overexpression of the E2F-1 transcription factor which induces an accumulation of cells in S-phase (Mueller *et al*., 1997).

A different experiment is shown in Figure 7B. Transfection of a UBPY sense plasmid accompanied by selection of the transfected clones with a neomycin resistance marker resulted in a severe inhibition of cell growth compared with controls, probably as a result of the dramatic changes in protein ubiquitination shown above (Figure 6). Similar effects were seen upon transfection of a mutated (Cys786Ala) UBPY construct, which also subverted protein ubiquitination (data not shown).

In conclusion, UBPY appears to play a critical role in the maintenance of the overall protein ubiquitination status. Acute alterations of its function can result in discrete effects on cell proliferation that reflect the particular time in the cell division cycle at which they occur.

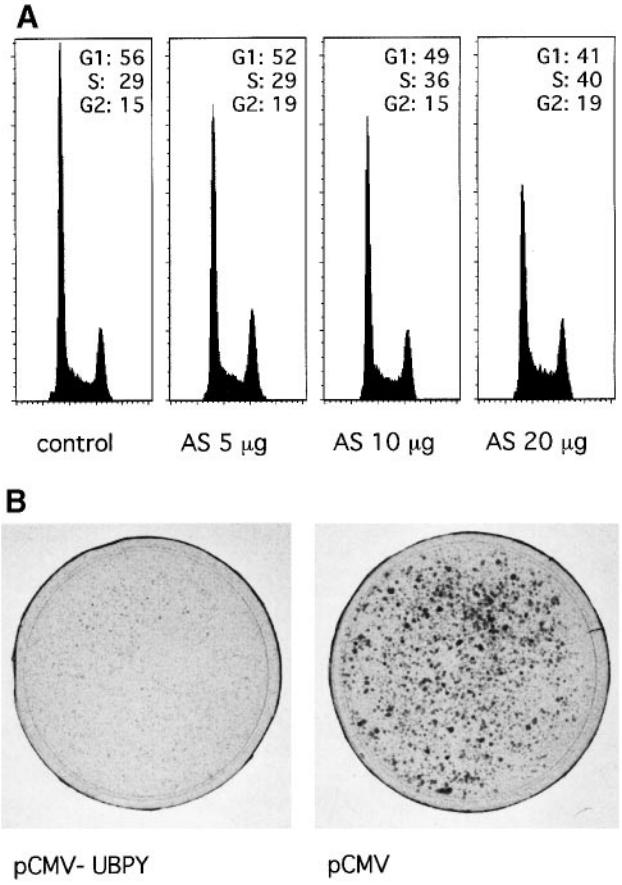

Fig. 7. Effects of altering UBPY expression on cell proliferation. (**A**) Dose–effect antisense experiment. U2OS cells were co-transfected with 2 μg of GFP plasmid and 20 μg of the pCMV vector (control), 15 μg of the same empty vector and 5 μg of PCMV–UBPY antisense construct, 10 μg of the same empty vector and 10 μg of PCMV–UBPY antisense construct, or 20 μg of PCMV–UBPY antisense construct. Propidium iodide staining of GFP-positive gated cells versus cell number is shown. Also indicated are the percentages of cells in the different phases of the division cycle. (**B**) Colony-forming assay. A total of 8×10^5 U2OS cells were plated in a 10 cm dish and transfected with either 20 μg of control empty pCMV vector or of the same vector containing the UBPY sequence in the sense orientation (PCMV–UBPY). Drug-resistant colonies (15 days after transfection) stained with crystal violet are shown.

Discussion

We have shown that in human cells, an antiserum generated against a recombinant UBPY fragment recognizes a protein doublet of M_r 130 000, in good agreement with the predicted UBPY mol. wt of 127 500 Da. The UBPY bands were enhanced upon transfection of a sense UBPY cDNA and, most importantly, were lowered in abundance upon transfection of an antisense cDNA construct. UBPY was able to remove ubiquitin from ubiquitin adducts. Its expression was undetectable upon serum starvation of normal human fibroblasts, and the protein reappeared upon re-stimulation, in mid G_1 coincident with the accumulation of cyclin D1. UBPY levels were reduced in non-immortalized cells as they reached confluence and arrested in G_0, while they remained high and even increased in transformed cells.

To date, no other studies have demonstrated the effects of down-regulating a UBP in mammalian cells. We have been able to inhibit UBPY accumulation using an antisense

cDNA vector and could demonstrate that G_0-arrested cells were prevented from entering S-phase after serum stimulation. Surprisingly, we found that de-regulation of UBPY accumulation generates a substantial derangement of the overall cell protein ubiquitination. Our data indicate that UBPY plays a general role in the ubiquitin pathway. One possible way to exert this function could be through the recycling of ubiquitin–tree peptide remnants which are generated after ubiquitinated proteins are cleaved by the proteasome. The consequences of inhibiting cleavage of these remnants by decreasing the cellular levels of UBPY or by expressing a Cys786 mutant which we know can avidly bind polyubiquitinated species (S.Naviglio and G.F.Draetta, unpublished) are consistent with our findings of an increase in the level of ubiquitinated species, probably due to the clogging of the proteasome by the ubiquitin remnants. A similar biochemical function has been proposed for the yeast Doa4 UBP (Papa and Hochstrasser, 1993). UBPY could exert a similar function, or could indirectly affect this function by controlling the ubiquitination state of one or more proteins which themselves control the activity of the proteasome.

What specific defects have been identified so far in cells lacking or carrying an altered UBP? In *Drosophila*, the product of the *fat facets* (*Faf*) gene encodes a UBP. *Faf* is required for *Drosophila* eye development and ovarian function (Fischer-Vize *et al*., 1992). It has been demonstrated that the *Faf* phenotype can be rescued either by expressing a fully functional FAF UBP, or by down-regulating proteasome function (Huang *et al*., 1995). This would argue that FAF protein antagonizes the proteasome because of its ability to remove ubiquitin from target substrates. In yeast, several UBPs have been identified. The yeast UBP1, UBP2 and UBP3 proteins are all able to cleave ubiquitin fusion proteins and polyubiquitin chains. While a triple disruption of yeast *UBP1, UBP2, UBP3* does not result in obvious growth defects (Baker *et al*., 1992), UBP3 protein has been found to interact with SIR4, a protein required for transcriptional silencing at the mating type locus. Yeast cells lacking *UBP3* have in fact an improved ability to activate silencing, suggesting that UBP3 is an inhibitor of silencing (Moazed and Johnson, 1996). A *Drosophila* UBP has also been implicated in transcriptional silencing (Henchoz *et al*., 1996). The yeast *DOA4* gene, also called *UBP4*, was identified due to its ability to restore degradation of the MATα2 transcription factor (Papa and Hochstrasser, 1993). Yeast lacking *DOA4* show slow growth and DNA repair defects and overexpression of a dominant-negative *DOA4* Cys→Ser mutant causes a slowing down in cell growth.

In mammalian cells, several UBPs have been identified. The Tre213-ORF2 protein was discovered as the product of a genetic rearrangement in human cells transfected with DNA from Ewing sarcoma cells and was shown to share structural similarities with the yeast DOA4 protein (Huebner *et al*., 1988). The Tre213-ORF2 protein has the hallmark signatures of a UBP, and it can hydrolyze ubiquitin fusion proteins (Nakamura *et al*., 1988, 1992; Papa and Hochstrasser, 1993). Given that a construct comprising a truncated, inactive form of Tre213-ORF2 was able to transform cells in culture, the hypothesis has been made that Tre213-ORF2, by removing ubiquitin from a growth-suppressing protein, enhances its activity and that the mutant protein could counteract these effects, thereby enhancing cell proliferation.

The DUB-1 UBP is the product of a cytokine-inducible gene (Zhu *et al*., 1996, 1997). Its mRNA and protein are induced very rapidly in response to interleukin-3 (IL-3) addition to a murine lymphocyte line, and then quickly down-regulated as cells progress into S-phase. The continuous expression of a wild-type DUB-1 protein, but not of a Cys→Ala mutant, causes cell cycle arrest in the G_1 phase of the division cycle, suggesting that its down-regulation in G_1 is essential for cell cycle progression. The same group has identified a second gene product, DUB-2, which is induced upon IL-2 addition to CTLL cells (Jaster *et al*., 1997). The mouse *UNP* gene was identified because of its proximity to a retroviral insertion site (Gupta *et al*., 1993). Cells transfected with *UNP* cDNA cause tumors in athymic mice (Gupta *et al*., 1994). The human *UNP* mRNA was also found overexpressed in human small cell lung primary carcinomas and cell lines (Gray *et al*., 1995). Recently, a further human UBP has been identified due to its ability to interact with Vmw110, an immediate early protein of herpes simplex virus type 1 (Everett *et al*., 1997). A gene encoding a novel human UBP has also been found on the X chromosome within a region involved in X-linked retinal disorders and found to be highly expressed in retina (Swanson *et al*., 1996).

As far as UBPY is concerned, our data indicate that this protein has a critical regulatory function which appears to be non-redundant with other UBPs we know to be expressed in the cell lines under study (C.Soncini, F.Goubin, S.Naviglio and G.F.Draetta, unpublished). As happens for other critical cellular components, the acute subversion of their function can result in specific phenotypes. We show that microinjection of antisense UBPY cDNA in quiescent human cells will prevent S-phase entry. A similar experiment performed in growing osteosarcoma cells will instead determine an accumulation of cells in S-phase. These apparently opposing effects can be explained by the fact that the ubiquitin system plays a role in many biochemical cascades required for cell division (for review see Pagano, 1997) and that at any given time an alteration of proteasome function can have discrete effects that will depend on the cell cycle phase at which the alteration occurs. One other probable explanation for these phenomena is that, compared with normal cells, the lack of G_1 checkpoint function in transformed cells makes them less sensitive to UBPY down-regulation early in the cell cycle. Further investigation will be necessary to clarify this issue.

Whether preferential substrates of UBPs exist is still an open question. The existence of 16 UBP family members in yeast would indicate that they have either distinct substrates or distinct upstream regulatory mechanisms that control their activity selectively. In human cells, we have been able to detect expression of different members of this family in the same cell type. We also know that in primary tissues certain of these enzymes show a remarkable tissue-specific expression and they can be over-expressed in human tumors (M.Capra and G.F.Draetta, in preparation). Here we demonstrate that inhibition of a cellular UBP can have a striking effect on cell proliferation. UBPs are cysteine proteases and as such are amenable to

the development of specific chemical inhibitors. The selective inhibition of any member of this family which showed a tissue- and/or tumor-specific expression could be considered as a strategy for the development of novel pharmaceutical agents.

Materials and methods

DNA cloning

The UBPY cDNA clone was first identified in the human myeloblast cell line KG-1. The entire UBPY cDNA sequence (bases 1–4359) was submitted (13 April, 1994) to the DDBJ/EMBL/GenBank databases by Nomura *et al*. (accession No. D29956). The UBPY coding sequence extends between bases 318 and 3674 and is predicted to encode a 1118 amino acid protein. A 3539 bp fragment (bases 226–3765) was cloned in the pBscSK+ expression vector in a *Hin*dIII site, then was cut out from this vector by *Xho*I–*Cla*I digestion and subcloned in the LTR-2 expression vector in the same sites (*Xho*I–*Cla*I). The fragement comprising bases 318–3674 was amplified by PCR, inserting 5′ and 3′ *Bam*HI sites, and subsequently cloned in a pCMV-neo-Bam CMV expression vector, in the sense or antisense orientation.

UBPY site-directed mutagenesis

The Cys786Ala mutation was introduced into UBPY by a two-step PCR-based approach. Oligodeoxynucleotides 5′-GGAAATACTGCTTATATG-AAC-3′ and 5′GTTCATATAAGCAGTATTTCC-3′ were used to mutate the Cys786 codon. Oligodeoxynucleotides 5′-CAACACTGTTCATAT-GTACC-3′ and 5′-CCGGGGATCCTTATGTGGCTACATCAGTTA-3′ served as flanking primers. PCMV-neo-Bam UBPY was used as a template. The second set of PCRs used the flanking primers and the two initial PCR products. The resulting DNA fragments were digested with *Nde*I and *Bam*HI (the *Nde*I restriction site was present constitutively in the UBPY sequence, while the *Bam*HI site was introduced into the second flanking primer) and gel purified. The 5′ *Nde*I–*Bam*HI 3′ mutated DNA fragment was used to replace the corresponding wild-type fragment and cloned as a three piece ligation into the PCMV–UBPY plasmid. The mutant construct was verified by dideoxynucleotide DNA sequencing.

Recombinant protein production

The full-length GST–UBPY and the GST–UBPY 9–188 fusion proteins were obtained by recombinant PCR of the appropriate fragment from the UBPY cDNA followed by cloning in the expression vectors pGEX-KG (*Bam*HI–*Eco*RI sites) for GST–UBPY and pGEX-KT (*Xba*–*Xho* sites) for GST–UBPY 9–188, in-frame with the GST moiety. The GST–UBPY protein contains the full-length UBPY protein without the initiator methionine (amino acids 2–1118). The GST–UBPY 9–188 protein contains the N-terminal portion of the UBPY cDNA between amino acids 9 and 188. Purification of the recombinant fusion proteins from bacterial supernatants was performed on glutathione–Sepharose according to the manufacturer's directions (Pharmacia).

Preparation and purification of rabbit polyclonal anti-UBPY antibodies

Polyclonal antibodies specific for the UBPY gene product were generated against the recombinant GST–UBPY 9–188 protein in New Zealand rabbits. Affinity-purified antibodies were obtained from total serum by affinity chromatography using GST–UBPY 9–188 conjugated to Amino-link (Pierce).

Extracts preparation, immunoprecipitation and immunoblotting

Cell extracts were prepared as described (Pagano *et al*., 1995b). Briefly, 3–5 volumes of lysis buffer [50 mM Tris–HCl pH 7.4, 0.25 M NaCl, 0.5% NP-40, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT)] were added to pelleted cells. The following protease inhibitors were also added: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, 1 μg/ml of aprotinin and 2 μg/ml of pepstatin. After incubation on ice for 30 min, samples were centrifuged at 14 000 r.p.m. in an Eppendorf microcentrifuge for 15 min at 4°C and the supernatant (NP-40 total extract) was recovered. Aliquots were taken for protein quantification, using the method of Bradford (1976). Immunoprecipitation and immunoblotting experiments were performed as described (Pagano *et al*., 1995b). Typically, we employed 10–50 μg of lysate protein for direct immunoblotting and 0.3–2 mg of lysate protein for immunoprecipitation/immunoblotting experiments. Filters were subjected to immunoblotting using the ECL (Amersham) detection system according to the manufacturer's directions.

Cell culture and cell synchronization

Human lung WI-38 fibroblasts, mouse NIH 3T3 fibroblasts, cervical carcinoma C33A and osteosarcoma U2OS cell lines were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 100 U/ml of penicillin, 1 μg/ml of streptomycin and 10% fetal calf serum (FCS) for WI-38 and NIH 3T3 cells, or 10% bovine calf serum (BCS) for C33A and U2OS cells. For synchronization by serum starvation, WI-38 fibroblasts (at 30–40% density) were made quiescent by culturing them for 3 days in DMEM containing 0.5% FCS. They were then given 10% FCS to induce synchronous entry into the cell cycle (Pagano *et al*., 1994). Cell cycle phases were monitored by flow-cytometry (see below).

Deubiquitination assays

Protein ubiquitination was generated as described (Pagano *et al*., 1995b) with some modifications. RRL (Promega) was used as the source of both ubiquitinating enzymes and substrate proteins, and was incubated at 37°C for 5–10 min in 300 μl of ubiquitination mix [final concentration 33% (v/v) RRL, 50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 2 mM DTT, 20 μg of added ubiquitin (Sigma), 1 mM ATPγS, 50 μM LLnL]. The reaction was stopped by the addition of NEM (20 mM final concentration). Sample was then centrifuged and washed several times, at 3200 r.p.m. for 20 min in a Centricon-3 concentrator (Amicon) to remove NEM. Aliquots of the ubiquitinated lysate (40 μg of proteins) were used as substrate for de-ubiquitinating activity: 0.5–1 μg of GST–UBPY were incubated at 37°C for 5–20 min in 20 μl of de-ubiquitination mix (40 μg of *in vitro* ubiquitinated proteins, 50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 2 mM DTT). Where indicated, GST–UBPY was pre-incubated with NEM to block its active site cysteine. Reactions were stopped with Laemmli buffer, boiled, resolved by SDS–PAGE (8%) and transferred to nitrocellulose. Filters were subjected to immunoblotting with anti-ubiquitin antiserum (Sigma) diluted (1:100) in 5% dry fat-free milk in TBS/0.05% Tween-20, and incubated at 4°C overnight.

UBPY de-ubiquitinating activity was also tested using a linear hexamer of HA-tagged ubiquitin (Ub6) as a substrate. The cDNA sequence encoding HA-tagged ubiquitin was excised from the parental plasmid pMT123 (Treier *et al*., 1994), and inserted into pET23 bacterial expression vector to transform BL21 bacteria. Methods for expressing and purifying polyubiquitin chains in bacteria have been described previously (Jonnalagadda *et al*., 1987). Purification of the recombinant Ub6 from the bacterial supernatant was performed on an S200 16/60 column, and the cleanest fractions were pooled, concentrated and used as substrate for UBPY de-ubiquitinating activity. To test this activity, UBPY immunoprecipitations from cell extracts were washed in lysis buffer (see above) with or without 10 mM NEM and washed further twice in the reaction buffer (50 mM Tris–HCl pH 8.3, 5 mM $MgCl_2$, 2 mM DTT), again with or without 10 mM NEM, and finally incubated at 37°C for 10–30 min in 90 μl of the same reaction buffer containing 20 ng/μl Ub6. At each time point, after spinning to pellet the beads, aliquots of the reactions were mixed with Laemmli buffer, boiled, resolved by SDS–PAGE (12%) and transferred to nitrocellulose. Filters were subjected to immunoblotting with monoclonal anti-HA antibodies to detect reaction products using the ECL (Amersham) system.

BrdU incorporation

Cells grown on glass coverslips were washed twice with phosphate-buffered saline (PBS), fixed for 10 min at room temperature with 4% paraformaldehyde in PBS, washed once in PBS and permeabilized with 0.25% Triton X-100 in PBS for 5 min at room temperature. Cells were then incubated for 20 s at room temperature in 50 mM NaOH. After three washes with PBS, cells were incubated for 30 min with monoclonal anti-BrdU (Becton Dickinson). After three further washes, cells were incubated for 30 min with Cy3-conjugated anti-mouse antibodies (Jackson Immunoresearch Laboratories) and then washed three times; counterstaining for DNA was performed by adding 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) into the final PBS wash. The coverslips were then washed once in distilled water and dried. The dried coverslips were mounted on slides with Mowiol and analyzed with a fluorescence microscope (Leitz Aristoplan). A JVC KYF55BE three-color digital video camera was used to obtain digitized images, which were then analyzed with the Image Grabber 24 1.2 software (Neotech).

Transfection experiments

Cells (8×10^5/plate) were split and were co-transfected 20 h later with calcium phosphate precipitates of 22 μg of plasmid DNA (ratio 10:1 between plasmid of interest and GFP plasmid) for each 100 mm dish. After 24 h, the cells were washed twice with PBS, and incubated with fresh 10% serum-containing medium. At 24–48 h after removal of DNA precipitates, cells were washed twice with PBS, rinsed off the plates with 0.05% trypsin, 0.2% EDTA in PBS, collected in 10% serum-containing medium and pelleted. Cell pellets were washed twice with PBS, divided into two aliquots and processed for immunoblotting and cell cycle analysis. For cell cycle analysis, the washed pellets were fixed in cold 70% methanol in PBS, pelleted and washed in 1% bovine serum albumin (BSA) in PBS. Fixed and washed cells were pelleted and stained in a solution of 50 μg/ml of propidium iodide and 100 μg/ml of RNase A for 1–2 h at 37°C. Flow-cytometry analysis was performed on a Becton-Dickinson FACScan, and data from 50 000–100 000 cells per sample were analyzed with the ModiFIT Cell Cycle Analysis software. A gate was set to select GFP-positive cells with a fluorescence at least 50-fold stronger than that in the negative non-transfected cells. The propidium iodide signal was used as a measure of DNA content and hence cell cycle stage. The DNA histograms referring to GFP-positive cells each contain data from 10 000–20 000 cells.

Colony formation assay

U2OS cells (8×10^5) were plated in a 10 cm dish and transfected by calcium phosphate precipitation either with 20 μg of control empty pCMV vector or of the same vector containing the UBPY sequence in the sense orientation. After 24 h, the cells were washed twice with PBS and incubated with fresh 10% serum-containing medium. At 24 h after the removal of DNA precipitates, cells were washed twice with PBS, rinsed off the plates with 0.05% trypsin, 0.2% EDTA in PBS, collected in 10% serum-containing medium and then split at different dilutions and subcultured into medium containing 500 μg/ml G418. Drug-resistant colonies appeared 10–15 days later and were either isolated for further analysis or counted after crystal violet staining.

Microinjection experiments

Cell monolayers growing on glass coverslips (at ~60% density) were microinjected with an automated microinjection system (AIS, Zeiss) connected with an Eppendorf injector. All microinjection experiments were carried out in 3.5 cm Petri dishes containing 5 ml of freshly added growth medium. Cells were injected at a pressure between 50 and 150 hecta-Pascal (hPa). The computer settings for injection were angle 45°, speed '10' and time of injection 0.1 s. Using this system, the percentage of successfully microinjected cells was >50%. pCMV-neo-Bam empty vector or the same vector containing UBPY full-length cDNA in the 3′–5′ antisense orientation were injected at a concentration of 50 ng/ml in the presence of a microinjector marker (GFP plasmid 20 ng/ml). Immediately after injection, fresh growth medium was added and supplemented with BrdU (Sigma) at a final concentration of 100 μM. Cells were fixed 24 h after serum re-addition. GFP detection by fluorescence microscope to identify microinjected cells, and BrdU incorporation (see above) were utilized to determine the effects of plasmid microinjection.

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