Structural and functional characterization of the USP11 deubiquitinating enzyme, which interacts with the RanGTP-associated protein RanBPM

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RanBPM is a RanGTP-binding protein required for correct nucleation of microtubules. To characterize the mechanism, we searched for RanBPM-binding proteins by using a yeast two-hybrid method and isolated a cDNA encoding the ubiquitin-specific protease USP11. The full-length cDNA of USP11 was cloned from a Jurkat cell library. Sequencing revealed that USP11 possesses Cys box, His box, Asp and KRF domains, which are highly conserved in many ubiquitin-specific proteases. By immunoblotting using HeLa cells, we concluded that 921-residue version of USP11 was the predominant form, and USP11 may be a ubiquitous protein in various human tissues. By immunofluorescence assay, USP11 primarily was localized in the nucleus of non-dividing cells, suggesting an association between USP11 and RanBPM in the nucleus. Furthermore, the association between USP11 and RanBPM *in vivo* was confirmed not only by yeast two-hybrid assay but also by co-immunoprecipitation assays using exogenously expressed USP11 and RanBPM. We next revealed proteasome-dependent degradation of RanBPM by pulse–chase analysis using proteasome inhibitors. In fact, ubiquitinated RanBPM was detected by both *in vivo* and *in vitro* ubiquitination assays. Finally, ubiquitin conjugation to RanBPM was inhibited in a dose-dependent manner by the addition of recombinant USP11. We conclude that RanBPM was the enzymic substrate for USP11 and was deubiquitinated specifically.

Key words: isopeptidase, nuclear protein, RanBPM-binding protein, ubiquitin-specific protease, UHX1.

INTRODUCTION

Proteolysis is one of the principal mechanisms whereby cellular protein levels are regulated. Proteolysis is involved in a variety of cellular functions, particularly cell-cycle regulation, because cell-cycle progression frequently is controlled by the degradation of regulatory proteins. In eukaryotes, proteolysis is triggered by ubiquitination of target proteins. Polyubiquitinated proteins are recognized and degraded by the 26 S proteasome [1,2]. Ubiquitin is a highly conserved 76 amino acid protein [3]. The conjugation of ubiquitin to protein is a highly regulated process that is controlled by a cascade of enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [4]. Among these enzymes, ubiquitin ligases interact directly with substrate proteins and thereby play central roles in substrate specificity. For example, ubiquitin conjugation to the cyclin-dependent kinase inhibitor $p27^{kip1}$ [5,6] and the transcriptional factor E2F-1 [7] was regulated specifically by the SCF^{skp2} ubiquitin ligase during the S phase of the cell cycle. This ligase therefore plays key roles in the entry to S phase [8,9].

Deubiquitinating enzymes are ubiquitin-specific thiol-proteases that cleave ubiquitin from its conjugate. Some deubiquitinating enzymes appear to perform an editing function, which controls the fidelity of the ubiquitin-conjugation process, thus preventing inappropriate degradation of cellular proteins. Two families of deubiquitinating enzymes have been identified on the basis of their *in vitro* activities and sequence similarities [10–12]. The ubiquitin C-terminal hydrolases are small (approx. 25 kDa) thiol-proteases that share amino acid sequence identity and cleave esters and amides from the C-terminus of ubiquitin. The other family of deubiquitinating enzymes is the ubiquitin-specific protease (UBP) family. Proteins in this family differ in length but possess conserved domains such as the Cys box and the His box. Many UBPs have been identified because they contain these conserved domains, and this situation suggests that substrate specificity is controlled by the heterogeneous regions [10,12,13]. USP11 was originally isolated and characterized in 1996, with the name ubiquitin C-terminal hydrolase on the X chromosome (UHX1) [14]. In that report, deubiquitinating activity was not characterized, but UHX1 was supposed to be a UBP because the protein contained a Cys box and a His box in its structure. UHX1 was mapped to chromosome Xp21.2–p11.2, a locus that may be related to hereditary X-linked retinal disease. Widespread tissue expression of UHX1 with 5–10-fold increased expression in the retina was shown [14]. Although UHX1 was proposed as a candidate gene for retinal disease in light of these findings, recent examination of 43 patients with X-linked retinal disease revealed no mutations in the UHX1 sequence [15]. Because the family of UBP is large and complex, a systematic nomenclature for human UBPs was proposed [16]. These enzymes were recently renamed USPs, and at least 28 USPs have been reported. UHX1 was renamed USP11, consistent with the nomenclature proposed for human USPs by the Human Genome Organization (HUGO) Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature).

RanBPM recently was isolated by a yeast two-hybrid method. It was found to be a Ran-binding protein and was shown to be required for correct microtubule nucleation [17]. When truncated

Abbreviations used: cLL, clasto-lactacystin β-lactone; DTT, dithiothreitol; FCS, fetal calf serum; IPTG, isopropyl β-D-thiogalactoside; UBP, ubiquitin-specific protease; UHX1, ubiquitin C-terminal hydrolase on the X chromosome.

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The USP11 nucleotide sequence was deposited in the GenBank/EMBL/DDBJ Nucleotide Sequence Databases under accession no. AB073597.

RanBPM was overexpressed in green monkey kidney COS7 cells, the normal radial network of microtubules in those cells was disrupted. In addition, a recent study showed nuclear localization of RanBPM, suggesting additional roles of RanBPM as a Ran-binding protein in the nucleus [18]. To further characterize RanBPM, we screened a human fetal brain cDNA library by a yeast two-hybrid method using human RanBPM as bait and subsequently cloned USP11 as a RanBPM-binding protein. The full-length USP11 cDNA encoded 921 amino acids, and a Cys box, a His box, Asp and KRF domains were completely conserved in this sequence. The enzymic activity of USP11 was confirmed by cleaving ubiquitin-β-galactosidase in *Escherichia coli* cells. Further, USP11 bound specifically to RanBPM and inhibited its ubiquitination and degradation.

EXPERIMENTAL

Materials and antibodies

Peroxidase-conjugated ExtrAvidin, ubiquitin, leupeptin, PMSF, creatine phosphate, creatine kinase, gentamicin, isopropyl β-D-thiogalactoside (IPTG) and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, U.S.A.). EZ-Link Sulfo-NHS-LC-biotin was obtained from Pierce. Ubiquitin aldehyde, clasto-lactacystin β-lactone (cLL), benzyloxycarbonyl-Leu-Leu-Leu-aldehyde (MG132) and acetyl-Leu-Leu-Nle-aldehyde (MG101) were bought from Boston Biochem (Cambridge, MA, U.S.A.). An antibody to ubiquitin was obtained from MBL, Nagoya, Japan. The anti-T7·Tag monoclonal antibody was purchased from Novagen (Madison, WI, U.S.A.), and the anti-β-galactosidase antibody was obtained from Promega.

To generate the antiserum against USP11, the $(His)_6$-tagged C-terminus of USP11 was expressed after cloning the DNA fragment that encodes amino acids 798–921 into pET16b (Novagen) and transfecting the resulting plasmid into BL21(DE3) *E. coli* cells. The expressed fusion protein was purified using a His·Bind Resin column (Novagen) according to the manufacturer's protocol. The recombinant protein was mixed with Freund's adjuvant and injected into rabbits according to standard procedures. In addition, rabbit polyclonal antibody was raised against a $(His)_6$-tagged protein comprising the 321 C-terminal amino acids of RanBPM. Both sera were affinity-purified by using a CNBr–Sepharose 4B column (Amersham Bioscience) coupled with the appropriate recombinant protein.

Cell culture

HeLa cells and NIH/Swiss cells were grown in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS) and 50 mg/l gentamicin and were split twice weekly using trypsin-EDTA (Life Technologies, Bethesda, MD, U.S.A.). Sf9 insect cells were grown in IPL-41 insect medium (Life Technologies) containing 0.6 mg/ml Triptose Phosphate Broth (Difco, Sparks, MD, U.S.A.), 5% FCS and 50 mg/l gentamicin.

Yeast two-hybrid screen

The Matchmaker Two-Hybrid System 2 (Clontech, Palo Alto, CA, U.S.A.) was used according to the manufacturer's protocol. Using PCR-based strategies, we subcloned the sequence encoding the RanBPM SPRY domain into the pAS2-1 vector to generate a fusion protein with the GAL4 DNA-binding domain (pAS-RanBPM) [19]. This plasmid was used as bait to screen a human fetal brain cDNA library (Clontech) constructed in the pACT2 vector to generate fusion products with the GAL4 activation domain. Yeast cells were transformed by the lithium acetate method as described in the manufacturer's protocol. The yeast strain Y190 (*MATa*; *ura3-52*; *his3-200*; *ade2-101*; *lys2-801*; *trp1-901*; *leu2-3*; *112*; *gal4*Δ; *gal80*Δ; *cyh*^{r}*2*; *LYS2*::$GAL1_{UAS}$-$HIS3_{TATA}$-*HIS3*, *URA3*::GAL_{UAS}-$GAL1_{TATA}$-*LacZ*) was co-transformed with pAS-RanBPM as a bait vector and the cDNA library plasmids. We grew 5×10^6 transformants on selective minimal plates (SD medium lacking leucine, tryptophan and histidine) containing 35 mM 3-aminotriazole for 7 days at 30 °C. We picked 30 colonies and amplified each in 1.5 ml of liquid SD medium lacking leucine to eliminate the bait plasmid. Each clone then was mated with the CG-1945 strain (*MATa*; *ura3-52*; *his3-200*; *lys2-801*; *ade2-101*; *trp1-901*; *leu2-3*; *112*; *gal4-542*; *ga80-538*; *cyh*^{r}*2*; *LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3*, *URA3*::$GAL4_{17\text{-}mer(\times 3)}$-$CyC1_{TATA}$-*lacZ*) containing either pAS-RanBPM or the control pAS2 vectors, and then the β-galactosidase assay was performed according to manufacturer's protocol to confirm the specific associations. The plasmids were then column-purified and sequenced using primers complementary to pACT2.

Cloning a full-length cDNA of USP11

A cDNA library from human Jurkat cells was screened using a ^{32}P-labelled cDNA probe. To prepare the probe, pACT-USP11, one of the isolated clones, was digested with *Eco*RI and *Xho*I. The resulting 1.1 kb fragment was gel-purified, then labelled with [α-^{32}P]dCTP using the Random Primer DNA Labelling Kit ver. 2 (TaKaRa Biomedicals, Shiga, Japan). We screened 2×10^6 plaques from the Jurkat cell library (Clontech) and isolated 21 positive clones. The insert of clone 31b, which was the longest cDNA insert, was subcloned into the *Eco*RI site of Bluescript SK(−) (pBlueUSP11) for sequencing and further plasmid constructions.

Plasmid construct

To obtain pcDNA-USP11, pET32b-USP11 and pFastBacHTb-USP11, pBlueUSP11 was digested with *Eco*RI to release the insert. The resulting 3.2 kb cDNA was gel-purified and subcloned into the *Eco*RI site of pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.), pET32b (Novagen) and pFastBac HTb (Life Technologies). To obtain pEGFP-USP11, partial cDNA of USP11 (694–2763 bp) was subcloned into the *Eco*RI site of pEGFP (Clontech). To obtain pDual-T7RanBPM, T7RanBPM was digested with *Eco*RI and *Pst*I; the resulting two cDNA fragments (1.3 kb and 1.2 kb) were gel-purified and sequentially ligated into the *Eco*RI–*Pst*I site of pFastBac Dual (Life Technologies). pUb-Met-βgal and pUb-Arg-βgal were gifts from Dr T. Suzuki [20].

Sucrose gradient sedimentation

For the sedimentation assay on a linear sucrose gradient, HeLa cells were washed twice with PBS and collected by scraping. The cell pellet was resuspended and lysed in an equal volume of Hepes-buffered saline [50 mM Hepes (pH 7.4), 15 mM NaCl and 1 mM EGTA] containing 0.5% Triton X-100 and 1 mM DTT on ice for 10 min; the suspension then was centrifuged at 12000 ***g*** for 10 min at 4 °C. We used a Hoefer gradient maker (Amersham Bioscience) to prepare a 10 ml 5–40% linear sucrose gradient. We loaded 500 μl of soluble cell lysate on the sucrose gradient, which was centrifuged at 160000 ***g*** in a SW40.1 rotor (Beckman Instruments) for 10 h at 4 °C. A parallel gradient was run with the standards of thyroglobulin (19 S), catalase (11 S) and BSA (4.5 S). Fractions were subjected to immunoblotting using antibodies against RanBPM and USP11.

Assay for UBP activity

The UBP activity of USP11 was confirmed by using a previously established method [20]. In brief, BL21(DE3) *E. coli* cells harbouring pET32b-USP11 were transformed with pUb-Met-βgal or pUb-Arg-βgal. After overnight liquid culture in the presence of ampicillin and chloramphenicol, expression of recombinant USP11 was induced by the addition of IPTG. We then transferred 200 μl of each culture into a 1.5 ml microcentrifuge tube, and the tubes were centrifuged at 12000 ***g*** for 30 s. After removal of the supernatants, cells were resuspended in 30 μl of 1 × Laemmli sample buffer. The ubiquitin-β-galactosidase and protease-cleaved β-galactosidase in the lysate were analysed by immunoblotting using an anti-β-galactosidase antibody.

In vivo degradation assay

By using the GenePorter2 Transfection Reagent (Gene Therapy Systems, San Diego, CA, U.S.A.), exponentially growing COS7 cells were transiently transfected with pT7RanBPM plasmid DNA. After being cultured for 2 days in complete medium, cells were washed twice with PBS and then cultured for 1 h in methionine- and cysteine-free Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10 % dialysed FCS. After the 1 h starvation, cellular proteins were pulse-labelled with Tran^{35}S-Label (300 μCi/ml; NEN Life Science Products) in the starvation medium for 3 h and then were chased in complete medium containing cLL, MG132 or vehicle only. Cells were harvested, lysed in lysis buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % SDS, 10 mg/ml leupeptin and 1 mM PMSF] and subjected to immunoprecipitation using T7·Tag antibody-conjugated Protein G–Sepharose. Precipitates were analysed by immunoblotting using T7·Tag antibody. ^{35}S-Labelled T7-tagged RanBPM in precipitates was quantified by using autoradiography and a BAS2000 image analyser (Fuji Photo Film Co., Tokyo, Japan).

Baculovirus expression system

To obtain recombinant RanBPM and USP11, we used the Bac to Bac Baculovirus Expression System (Life Technologies). *E. coli* DH10Bac (Life Technologies) was transformed with pDual-T7RanBPM or pFastBacHTb-USP11 plasmid DNA, then selected on LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal (Life Technologies) and 40 μg/ml IPTG. White colonies were picked and amplified, and recombinant bacmids were extracted. Sf9 insect cells were transfected with the recombinant bacmid using Cellfectin (Life Technologies). After culturing for 4 days, the expression of recombinant RanBPM and USP11 was confirmed by immunoblotting analysis using antibodies against T7·Tag and USP11. Recombinant RanBPM and USP11 were purified from 5×10^9 infected cells by using T7·Tag antibody-conjugated Protein G–Sepharose and Talon Metal Affinity Resins (Clontech), respectively.

In vitro ubiquitination assay

Biotinylated ubiquitin- and proteasome-free cell extracts (S100Pr$^-$) were prepared using NIH/Swiss cells according to previously reported methods [21]. Baculovirus-expressed RanBPM was incubated with the S100Pr$^-$ in the presence of an ATP-regenerating system [50 mM Tris (pH 8.3), 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate and 0.2 units/ml creatine kinase] together with 2 μg/ml biotinylated ubiquitin, 100 μg/ml ubiquitin aldehyde, 2 mM DTT, 10 μg/ml leupeptin, 10 μg/ml PMSF and proteasome inhibitor mix (250 μM MG101, 250 μM MG132 and 25 μM cLL). The reactions were carried out at 25 °C for 1 h and terminated by adding an equal volume of 2 × Laemmli sample buffer. Samples were subjected to SDS/PAGE and blotted on to nylon membrane. Ubiquitinated T7-tagged RanBPM in each lane was visualized by using an anti-RanBPM antibody and horseradish peroxidase-conjugated ExtrAvidin (Sigma).

RESULTS

Isolation and identification of USP11

To identify the proteins interacting with the RanBPM SPRY domain, we performed a yeast two-hybrid screen in which 5×10^6 independent clones from a human fetal brain library were screened using the Y190 yeast strain, and 30 clones were isolated as candidates. These 30 candidates were studied further to confirm the association with RanBPM by using another yeast strain, CG1945, and 12 clones were sequenced. Among these, clone 7 (pACT-USP11) encoded sequence corresponding to USP11 [14]. The resulting product was the 124 C-terminal amino acids of USP11, which were fused in frame with the GAL4

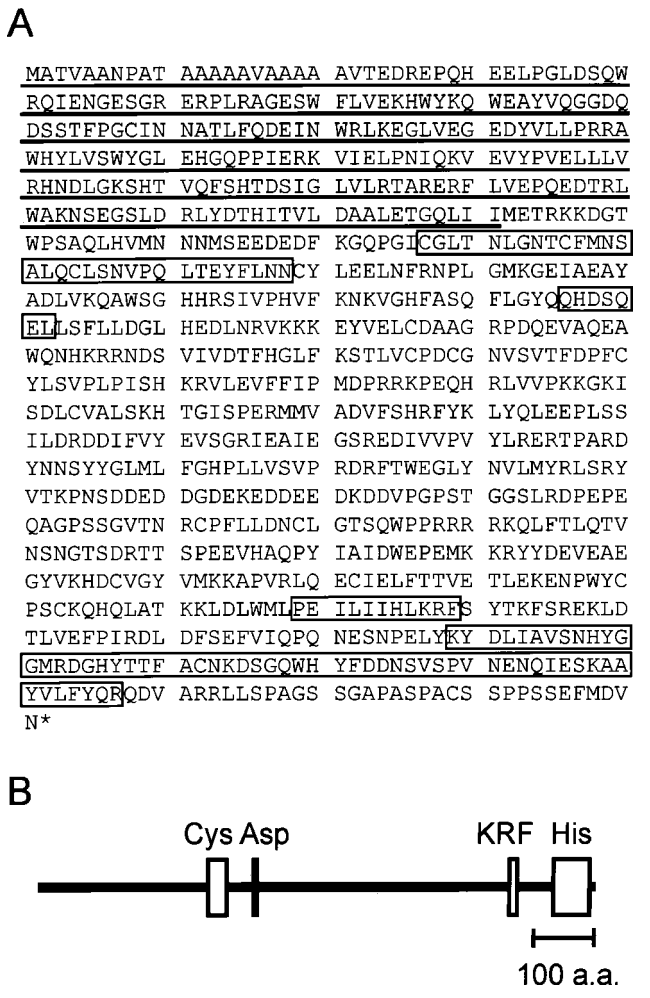

Figure 1 Identification of USP11

(**A**) Sequencing of clone 31b revealed that USP11 consists of 921 amino acids and possesses Cys box, Asp, KRF and His box domains. These domains are boxed. Newly identified sequence is underlined. (**B**) The structure of USP11 is shown schematically; a.a., amino acids.

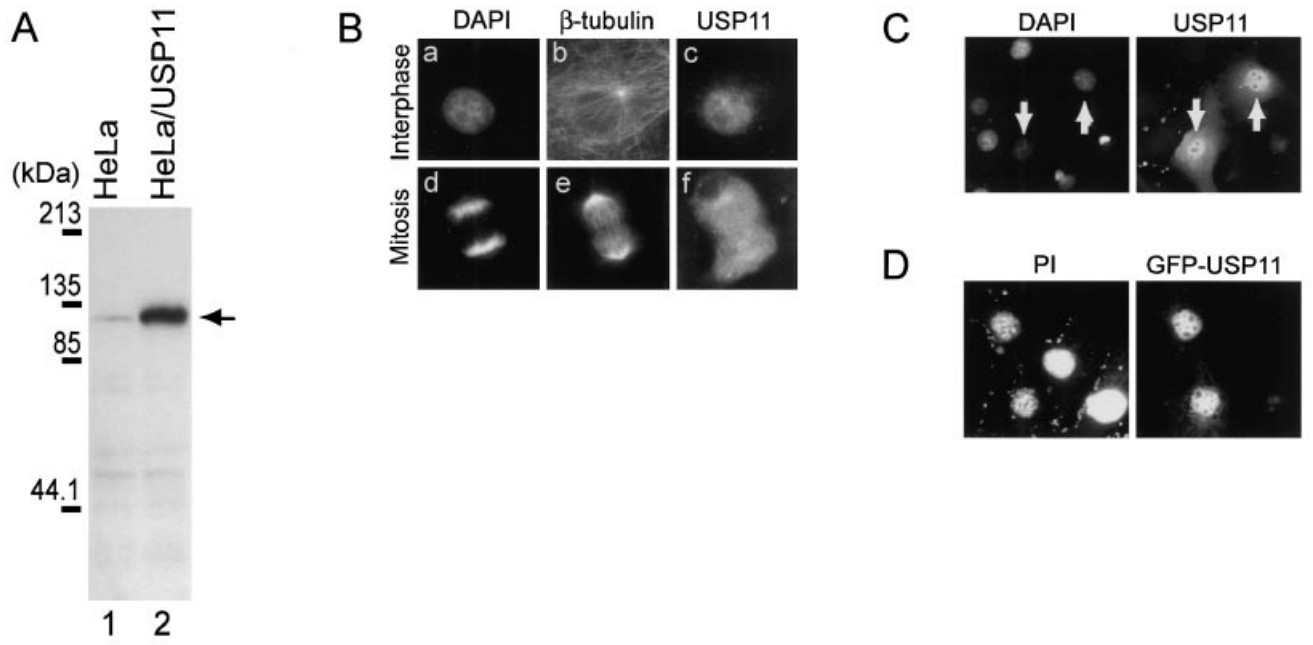

Figure 2 Characterization of USP11

(**A**) Exponentially growing HeLa cells were lysed and the proteins were separated on an 8% denaturing polyacrylamide gel (lane 1). HeLa cells were transfected with pcDNA-USP11 and, after a 2-day incubation, were lysed and separated as described (lane 2). The separated proteins were transferred to a nylon membrane and probed by using an anti-USP11 antibody. The arrow indicates the USP11-specific bands of 120 kDa. (**B**) Exponentially growing HeLa cells were subjected to double immunofluorescence staining with antibodies against β-tubulin (panels b and e) and USP11 (panels c and f). In interphase cells, USP11 staining is predominantly in the nucleus, which is revealed by DAPI (4,6-diamidino-2-phenylindole) staining (panel a). In mitotic cells, USP11 is spread throughout the cytoplasm (panel f). (**C**) HeLa cells transfected with pcDNA-USP11 were subjected to immunofluorescence analysis. Arrows indicate the nuclear distribution of exogenous USP11. (**D**) The intranuclear distribution of USP11 was also determined by fluorescence microscopy of HeLa cells transfected with pEGFP-USP11(694–2763bp). PI, propidium iodide.

activation domain. The 124 amino acids encoded a His box domain, which has been well characterized as a domain conserved among deubiquitinating enzyme family proteins. To isolate a full-length USP11 cDNA, we used a cDNA probe containing the 124 amino acids isolated in clone 7 to screen 2×10^6 independent clones of the Jurkat cell library; 21 positive clones were isolated. Clone 31b, which contained the longest USP11 cDNA insert, encoded the entire reported USP11 sequence.

The first report of USP11 described an open reading frame consisting of 2070 bp that coded for a predicted protein of 690 amino acids [14]. Sequencing of the clone 31b revealed that the cDNA was preceded by a 34 bp 5′-untranslated region. Surprisingly, the extended 5′-sequence contained an ATG codon, and the resulting open reading frame consisted of 2763 bp and encoded a predicted protein of 921 amino acids with a relative molecular mass of 105183 Da. The Cys box, His box, Asp and KRF domains of the original USP11 isolate were completely conserved in the newly isolated USP11 (Figure 1). Because there was no stop codon at the site upstream from the newly identified ATG codon, we repeated the library screening. Although another 42 positive clones were analysed, we did not isolate a cDNA clone extending from the 5′-end of clone 31b RanBPM cDNA.

To investigate the function of USP11, we raised a rabbit polyclonal antibody against the C-terminal 124 amino acids. The affinity-purified antibody recognized four protein bands of approx. 120, 55, 50 and 44 kDa in an immunoblot, using total extract from HeLa cells (Figure 2A, lane 1). When HeLa cells were transfected with pcDNA-USP11, the exogenous USP11 also was revealed as a band of approx. 120 kDa (Figure 2A, lane 2). We concluded that the 921-residue version of USP11 is one of the predominant forms of the protein. Although three other smaller proteins may be degradation products, some other spliced products could possibly correspond to the smaller proteins and possess functions. Several cell lines from various human tissues were subjected to immunoblotting, and the expression of USP11 was confirmed in each line (results not shown), suggesting ubiquitous expression of USP11.

We investigated the intracellular distribution of USP11 through immunofluorescence microscopy of HeLa cells using the affinity-purified polyclonal antibody. The cells were double-stained for USP11 and β-tubulin. As shown in Figure 2(B), USP11 primarily was localized in the nucleus (upper panels) of non-dividing cells and was found throughout mitotic cells (lower panels). When HeLa cells were transfected with pcDNA-USP11 (Figure 2C) and with pEGFP-USP11(694–2763bp) (Figure 2D), the exogenous USP11 predominated in the nucleus. Because the nuclear localization of RanBPM was reported recently [18], our finding suggested an association between USP11 and RanBPM in the nucleus. When cells were fixed with methanol and then double-stained, we did not detect any USP11 on centrosomes (results not shown).

Association between RanBPM and USP11 *in vitro*

We again performed yeast two-hybrid assay using the Y190 yeast strain. As shown in Figure 3(A), the USP11 was specifically associated with RanBPM but not with the GAL4 binding domain or p53 (as a control protein). The RanBPM was specifically associated with USP11 but not with the GAL4 activating domain or simian virus 40 large T-antigen (as a control protein). The results of a co-immunoprecipitation assay confirmed the association between RanBPM and USP11. We first performed the

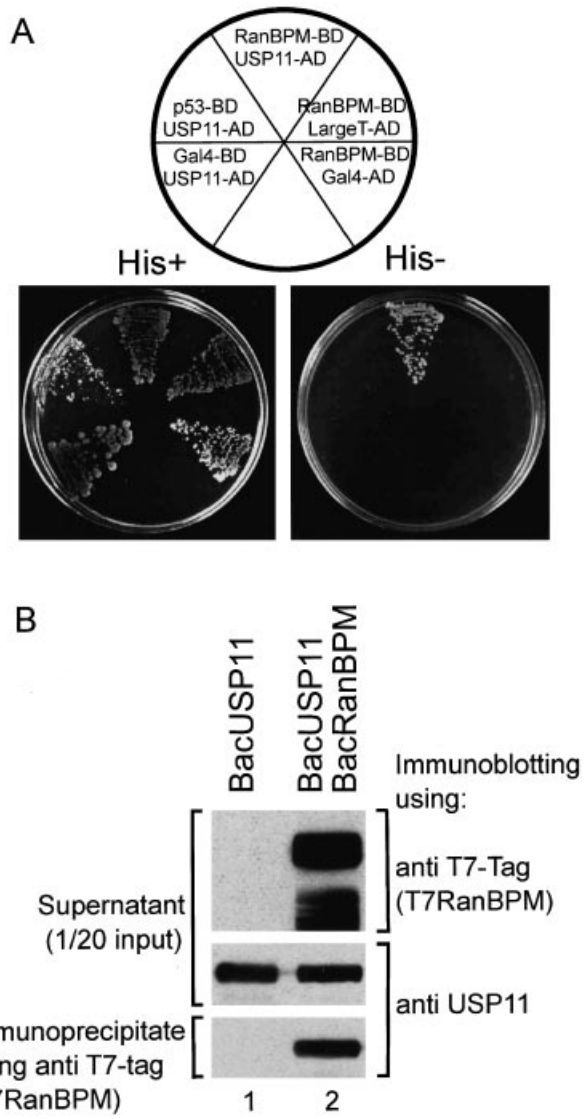

Figure 3 *In vivo* association between USP11 and RanBPM

(**A**) The yeast strain Y190 was transfected with the indicated plasmids and grown on SD medium lacking leucine and tryptophan for 4 days at 30 °C. Each picked colony was streaked on a selective minimal plate (His − ; SD medium lacking leucine, tryptophan and histidine) containing 20 mM 3-aminotriazole and a selective minimal plate containing histidine (His +). GAL4 DNA-binding domain (Gal4-BD), GAL4 activation domain (Gal4-AD), p53-binding domain (p53-BD) and simian virus 40 large T-antigen activation domain (LargeT-AD) were negative controls. BD, binding domain; AD, activation domain. (**B**) USP11 and T7-tagged RanBPM were overexpressed in Sf9 cells co-infected with the recombinant baculoviruses BacUSP11 and BacRanBPM (lane 2). The expression of exogenous USP11 and RanBPM was confirmed by immunoblotting using an anti-USP11 antibody (middle panel) and a T7·Tag antibody (upper panel), respectively. T7-tagged RanBPM was immunoprecipitated using T7·Tag antibody-conjugated Protein G–Sepharose. USP11 was detected in the precipitate (lower panel). As a negative control, Sf9 cells were infected with the recombinant baculovirus BacUSP11 alone (lane 1).

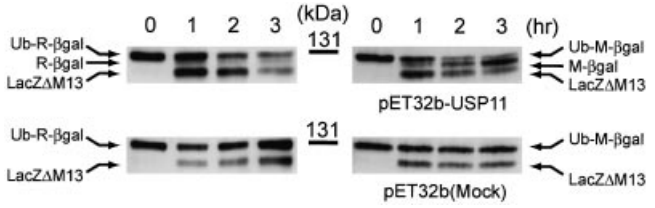

Figure 4 Deubiquitinating activity of USP11 in *E. coli*

The parental *E. coli* BL21(DE3) cells first were transformed with pET32-USP11, then with pUb-Arg-βgal or pUb-Met-βgal (upper panels). In a control experiment, pET32b was used as a mock vector in the co-transformation (lower panels). The cells were grown to late logarithmic-phase in 50 ml of LB medium. A 200 μl aliquot of each liquid culture was collected before IPTG induction (0 h) and at the indicated times after the induction. The harvested cells were then subjected to immunoblotting using an anti-β-galactosidase antibody. The arrows indicate the ubiquitin-β-galactosidase fusion proteins (Ub-R-βgal and Ub-M-βgal), the cleaved β-galactosidase moieties (R-βgal and M-βgal) and endogenous β-galactosidase (LacZ-M13) fragments.

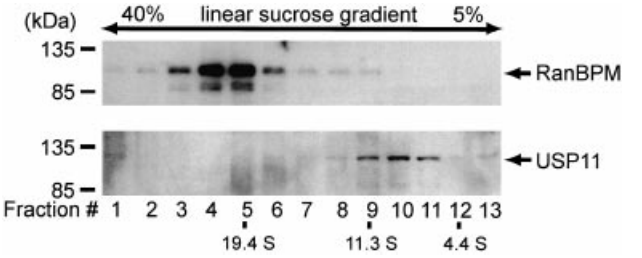

Figure 5 RanBPM but not USP11 is part of a large protein complex

Extract from HeLa cells was centrifuged through a linear sucrose gradient as described in the Experimental section. Fractions collected from the bottom upwards were subjected to immunoblotting using antibodies against RanBPM (upper panel) and USP11 (lower panel). Standards used were BSA (4.4 S), bovine catalase (11.3 S) and bovine thyroglobulin (19.4 S) and are indicated below the panels.

assay using endogenous RanBPM and USP11, but they did not co-immunoprecipitate (results not shown). Therefore, our antibodies were excellent for immunoblotting but insufficient for immunoprecipitation. We therefore performed the immunoprecipitation using a T7·Tag antibody and recombinant baculovirus-infected Sf9 cells, in which both USP11 and T7-tagged RanBPM were overexpressed under the control of the polyhedrin promoter (Figure 3B, lane 2, upper and middle panels). T7-tagged RanBPM was precipitated efficiently by T7·Tag antibody-conjugated Protein G–Sepharose, and USP11 co-precipitated in the pellet (Figure 3B, lane 2, lower panel). For a negative control, we infected Sf9 cells with USP11 recombinant baculovirus alone (Figure 3B, lane 1, upper and middle panels). These results agree with those obtained by the yeast two-hybrid method.

Deubiquitinating activity of USP11

Because its protease activity was not yet confirmed, we characterized this property of USP11 by applying a previously established method [20] (Figure 4). We first co-expressed USP11 and ubiquitin-Met-β-galactosidase in *E. coli*, in which USP11 hydrolysed ubiquitin-Met-β-galactosidase to free ubiquitin and Met-β-galactosidase. Ubiquitin-Met-β-galactosidase was converted into Met-β-galactosidase time-dependently after IPTG induction (Figure 4, upper right panel). When we co-expressed USP11 and ubiquitin-Arg-β-galactosidase in *E. coli*, USP11 hydrolysed ubiquitin-Arg-β-galactosidase to release Arg-β-galactosidase. Compared with Met-β-galactosidase, Arg-β-galactosidase is short-lived in *E. coli* [22,23]; therefore ubiquitin-

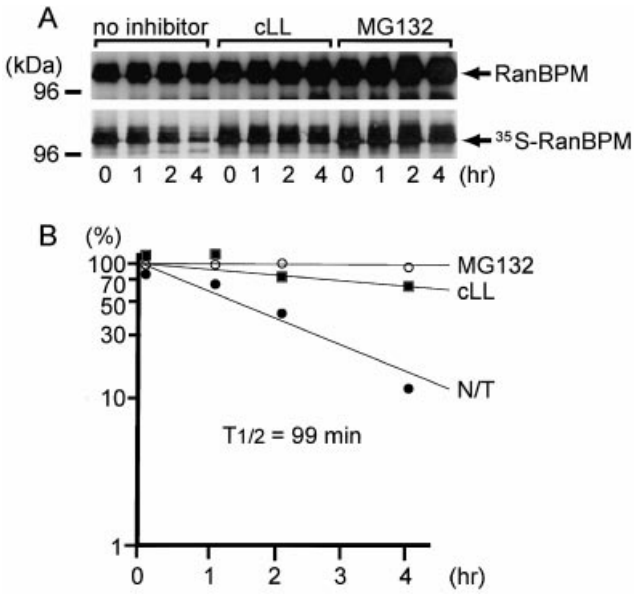

Figure 6 Proteasome-dependent degradation of RanBPM

T7-tagged RanBPM was pulse-labelled with [^{35}S]methionine *in vivo*, and the cells were chased as described in the Experimental section. After incubation with indicated proteasome inhibitor for the indicated time, cells were lysed and RanBPM was immunoprecipitated using a T7·Tag antibody. (**A**) RanBPM in the precipitate was analysed by immunoblotting using a T7·Tag antibody (upper panel). The radioactivity incorporated in RanBPM was quantified by autoradiography (lower panel) and a Bioimaging Analyser BAS2000. (**B**) Results from the BAS2000 are shown as the ratio of the radioactivity at the indicated time to that from the sample at time 0. In the absence of inhibitors, the ^{35}S-labelled RanBPM (●; N/T) was degraded rapidly, with a half-life of approx. 99 min. The proteasome inhibitors MG132 (○) and cLL (■) inhibited the degradation.

Arg-β-galactosidase disappeared time-dependently, and Arg-β-galactosidase was detected only faintly (Figure 4, upper left panel). In control experiments, a pET32b mock vector was used in the co-transformation (Figure 4, lower panel); no deubiquitinating activity was detected in these mock-transformed *E. coli*. These results clearly indicate the UBP activity of USP11.

Ubiquitin-proteasome-dependent degradation of RanBPM

Recent investigation revealed that RanBPM was a part of a large complex of proteins. Because partial purification using a gel-filtration method revealed that the RanBPM-containing complex was larger than 670 kDa [18], we examined the possibility that USP11 also was a component of this complex. To compare the sedimentation behaviour of USP11 with that of RanBPM, we applied a soluble extract from HeLa cells to a 5–40 % linear sucrose gradient and analysed the resulting fractions by immunoblotting using antibodies against RanBPM and USP11. RanBPM migrated as a 20 S protein (fractions 4 and 5; Figure 5, upper panel), the size of which probably corresponds to the large complex previously reported. In contrast, the concentration of USP11 peaked at fraction 10 (Figure 5, lower panel). Although the resolution of this assay was insufficient to determine whether USP11 is a monomer or part of a heterodimer, USP11 clearly is not part of the 20 S complex.

We then investigated whether RanBPM was an enzymic substrate of USP11. To elucidate this possibility, we examined the mechanism of RanBPM degradation. Exponentially growing COS7 cells were transfected with 2 μg of T7RanBPM plasmid DNA. After incubation for 2 days, the exogenous T7-tagged RanBPM was pulse-labelled *in vivo* with [^{35}S]methionine. The ^{35}S-labelled RanBPM was degraded rapidly, with an approximate half-life of 99 min (Figure 6). When cells were treated with cLL or MG132, the half-life was prolonged markedly. These results suggested that RanBPM is degraded by a ubiquitin-proteasome-mediated pathway. Both cLL and MG132 have been well characterized as proteasome inhibitors. However, MG132 is a leupeptin analogue, which also inhibits caspases and calpains [24,25]. In addition, lactacystin (and presumably its active metabolite cLL) also inhibits cathepsin A activity [26]. These

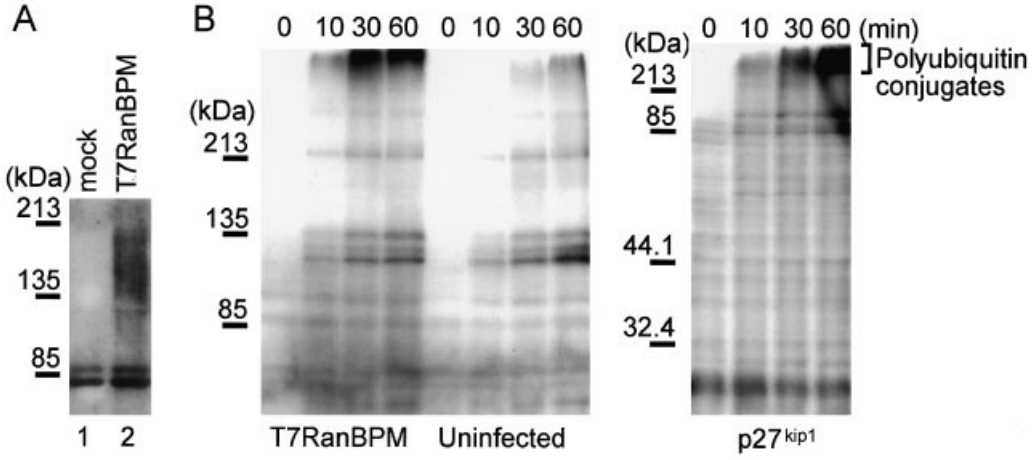

Figure 7 Ubiquitin conjugation of RanBPM

(**A**) Exponentially growing COS7 cells were transfected with T7RanBPM and pSRD mock vector. After incubation for 2 days, T7-tagged RanBPM was precipitated by a T7·Tag antibody-conjugated Protein G–Sepharose. The precipitates were subjected to immunoblotting using an anti-ubiquitin antibody. Ubiquitin conjugates were detected only in T7RanBPM-expressing cells (lane 2). (**B**) An *in vitro* ubiquitination assay was performed as described in the Experimental section. Baculovirus-expressed T7-tagged RanBPM and bacterially expressed glutathione S-transferase-tagged p27^{kip1} were subjected to this assay as substrates. After incubation for 0, 10, 30 or 60 min, ubiquitin conjugates were separated by SDS/PAGE and probed by ExtrAvidin. High-molecular-mass polyubiquitin conjugates of RanBPM (left panel) and p27^{kip1} (right panel) accumulated at the top of the gel in a time-dependent manner. As a negative control, the precipitate from Sf9 cell extracts treated with a T7·Tag antibody was used in this assay (middle panel).

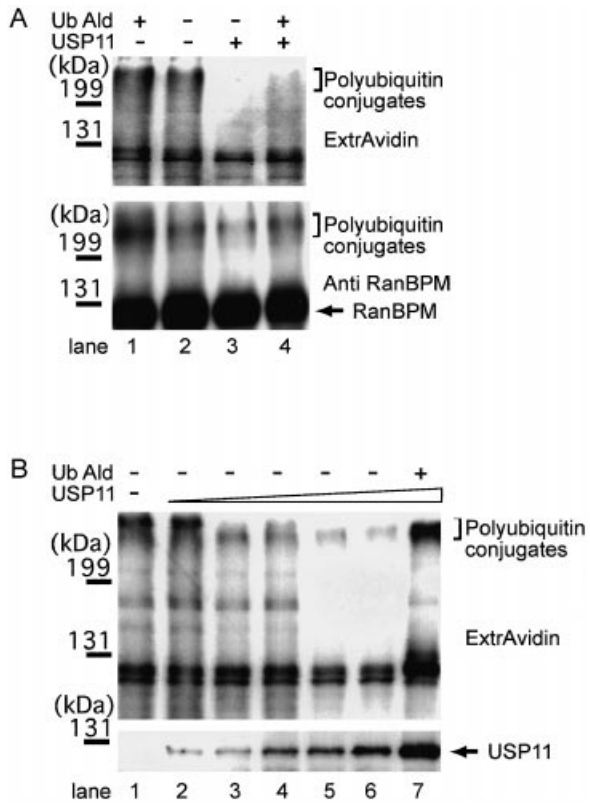

Figure 8 Specific deubiquitinating activity of USP11 on ubiquitin-conjugated RanBPM

An *in vitro* ubiquitination assay in which baculovirus-expressed T7-tagged RanBPM was the substrate was performed as described in the Experimental section. (**A**) Polyubiquitin-conjugated RanBPM was probed with ExtrAvidin (upper panel) and an anti-RanBPM antibody (lower panel). The polyubiquitination was suppressed by USP11 (lane 3), indicating its deubiquitinating activity. An inhibitor of deubiquitination, ubiquitin aldehyde (UbAld, 100 μg/ml), inhibited the USP11 enzymic activity (lane 4). (**B**) Polyubiquitin-conjugated RanBPM was probed by ExtrAvidin. The polyubiquitination was dose-dependently suppressed by USP11. The reaction mixture contained 0 (lane 1), 20 (lane 2), 40 (lane 3), 60 (lane 4), 80 (lane 5) or 100 ng (lanes 6 and 7) of USP11 and was incubated at 25 °C for 1 h.

results suggested that RanBPM is degraded by a ubiquitin-proteasome-mediated pathway.

To detect ubiquitin-conjugated RanBPM, we performed *in vivo* and *in vitro* ubiquitin-conjugation assays. COS7 cells were transfected with T7RanBPM. Then, T7-tagged RanBPM was immunoprecipitated using T7·Tag antibody-conjugated Protein G–Sepharose. Ubiquitin conjugates in the precipitates were analysed by immunoblotting using an anti-ubiquitin antibody. Compared with those in control cells, ubiquitin conjugates were enriched in cells overexpressing T7-tagged RanBPM (Figure 7A). We then expressed recombinant T7-tagged RanBPM in Sf9 cells infected with recombinant baculovirus. After precipitation with T7·Tag antibody, the precipitate was incubated with biotinylated ubiquitin and S100Pr$^-$ in the presence of an ATP-regenerating system. As shown in Figure 7, high-molecular-mass polyubiquitin conjugates of T7-tagged RanBPM and p27^{kip1} accumulated in a time-dependent manner. As a negative control, the precipitate from uninfected Sf9 cells treated with T7·Tag antibody was subjected to this assay. Although some unknown contaminating proteins appeared faintly after 30 or 60 min, we concluded that RanBPM is conjugated with ubiquitin and is degraded by a ubiquitin-proteasome-mediated pathway.

Deubiquitination of RanBPM by USP11

We examined whether the ubiquitin conjugation of RanBPM was inhibited by USP11. We performed an *in vitro* deubiquitination assay, which was similar to the ubiquitin-conjugation assay except that the inhibitor of ubiquitin hydrolases (ubiquitin aldehyde) [27] was omitted from the reaction mixture, and recombinant USP11 was added to it. Anti-RanBPM antibody and ExtrAvidin clearly labelled high-molecular-mass ubiquitinated RanBPM (Figure 8A, lane 1). The ubiquitination was inhibited slightly by contaminating deubiquitinating activities in the ubiquitin aldehyde-free reaction mixture (Figure 8A, lane 2). When USP11 was added to the mixture, ubiquitination was inhibited strongly (Figure 8A, lane 3), and ubiquitin aldehyde (100 μg/ml) inhibited the enzymic activity of USP11 (Figure 8A, lane 4). The deubiquitinating activity of USP11 on ubiquitin-conjugated RanBPM was dose-dependent (Figure 8B).

DISCUSSION

Identification and characterization of USP11

In the present study, we isolated a full-length USP11 cDNA. Sequencing revealed a 3217 bp cDNA encoding 921 amino acids, but no stop codon was detected in the 5′-untranslated sequence. Therefore, we thought that the USP11 mRNA might be translated from an unknown ATG start codon located further upstream. Indeed, when Brandau et al. [15] investigated the genetic structure of the USP11 gene on chromosome Xp11.2, the gene structure program XGRAIL2 predicted 21 exons and a 2889 bp open reading frame encoding 963 amino acids. To isolate a clone containing the additional upstream sequence, we screened another 4×10^6 independent clones of the Jurkat cell library, but we were unsuccessful in this regard. Furthermore, immunoblotting showed that the 921 amino acid exogenous USP11 migrated to the same position as the endogenous USP11. In light of these results, we concluded that the 921-residue version was the major form of USP11.

In a previous report, USP11 was characterized as a predicted protein of 690 amino acids that contained Cys box, Asp, KRF and His box domains [14]. To clarify whether our newly identified N-terminal sequence encoded any important domains, we searched for homologies between our sequence and those in the protein profile databases by using the basic local alignment search tool (BLAST) program. Although we could not find any similarities to established domains, amino acids in the newly identified sequence were highly homologous with those of deubiquitinating enzymes such as USP4, USP15 and USP20. Therefore, the N-terminus may be important for the deubiquitinating activity of USPs.

Next, we examined the cellular distribution of USP11. Although RanBPM originally was reported as a centrosome protein [17], our immunofluorescence analysis failed to reveal USP11 on the centrosome. Furthermore, we confirmed the nuclear distribution of USP11 in extensively repeated assays. Recently, Nishitani et al. [18] characterized the nuclear localization of RanBPM. We therefore supposed that USP11 interacts with RanBPM in the nucleus.

RanBPM is the specific substrate of USP11

We then investigated the functional relation between USP11 and RanBPM. Because RanBPM recently was confirmed to be a part of a large (> 670 kDa) protein complex [18], we first investigated whether USP11 was also a subunit of this complex. A sedimentation assay using a linear sucrose gradient showed that

RanBPM but not USP11 migrated as a 20 S protein, indicating that USP11 is not a part of the RanBPM-containing protein complex. Then we asked whether RanBPM is a specific substrate of the deubiquitinating enzyme USP11. Many UBPs have been identified in light of their conserved domains (including Cys and His boxes), and the enzymic activity of some of these proteins has been elucidated by the same standard methods that we used in the present study. Because the specificity of ubiquitination is regulated by the direct association between each ubiquitin ligase and its specific substrate, it is supposed that the binding of a UBP to its substrate also regulates the specificity of deubiquitination [10–12]. Many recent investigations have addressed the substrate specificity of ubiquitin ligase, but the mechanism underlying that of the deubiquitinating enzyme remains unknown. Because ubiquitin ligases and UBPs synergistically regulate protein degradation, investigations addressing deubiquitination will probably garner much interest.

A few recent investigations have delved into the binding of UBPs to their specific substrates. Using yeast two-hybrid screening, Gnesutta et al. [28] isolated a cDNA encoding the deubiquitination enzyme mUBPy as a binding protein of the Ras nucleotide exchange factor CDC25 Mm. They also revealed that exogenously expressed CDC25 Mm was ubiquitinated and that its co-expression with mUBPy decreases the ubiquitination. In addition, the half-life of the CDC25 Mm protein was increased considerably in the presence of mUBPy. Taya and co-workers purified an AF-6-interacting protein with a molecular mass of 220 kDa [29]. The peptide sequence of the protein was identical with that of the deubiquitinating enzyme Fam [29]. These investigators also found that AF-6 was ubiquitinated in culture cells and that Fam prevented its ubiquitination. In the present study, we characterized the binding of RanBPM to USP11 as an enzymic substrate. To understand the substrate-specific deubiquitinating system, similar future investigations will be important.

Biological significance of the association between USP11 and RanBPM

In the present study, we clarified that USP11 specifically associates with RanBPM and inhibits its ubiquitination and degradation. Now, we question the biological significance of USP11 regulating the degradation of RanBPM. RanBPM originally was cloned as a Ran-binding protein [17]. Ran has been well characterized as a molecule required for nuclear protein import and export, and recent studies revealed that Ran is also involved in spindle formation of mitotic and meiotic cells. Chromatin-induced spindle formation was severely repressed by depletion of the RanGDP–GTP exchange factor RCC1 in an *in vitro* system using *Xenopus* meiotic egg extract, and the addition of RanGTP dramatically reversed the repression. Because RanGTP does not stimulate microtubule polymerization from purified tubulin subunits, additional cofactors must be located downstream of RanGTP signalling and involved in RanGTP-dependent spindle formation [30–32]. Although importin-β was identified as one of the target molecules of RanGTP based on the evidence of its suppressive effects on spindle formation [33–35], recent investigations have revealed multiple potential target molecules in this signalling pathway [36,37]. Further, overexpression of RanBPM resulted in ectopic microtubule nucleation, and an antibody against RanBPM inhibited microtubule nucleation [17]. We therefore became greatly interested in possible involvement of RanBPM in the signalling pathway. When we began our study, our aim was to identify the molecules involved in spindle formation and microtubule nucleation. We now are investigating whether USP11 is involved in the signalling. We have overexpressed USP11 in several cell lines but have been unable to identify any effects of this overexpression on spindle formation, microtubule organization or the cell cycle. Further investigation into the biological effects of USP11 will be of interest.

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