Nuclear-Cytoplasmic Shuttling of the Oncogenic Mouse Unp/USP4 Deubiquitylating Enzyme*

The oncogenic deubiquitylating enzyme (DUB) Unp/USP4, which binds to the retinoblastoma family of tumor suppressor proteins, was originally described as a nuclear protein. However, more recent studies have shown it to be cytoplasmic. In addition, analysis of its subcellular localization has been complicated by the existence of the paralog Usp15. In this study, we resolved this controversy by investigating the localization of exogenously expressed Usp4 (using red fluorescent protein-Usp4) and of endogenous Usp4 (using highly specific antibodies that can distinguish Usp4 from Usp15). We found that by inhibiting nuclear export with leptomycin B, both exogenous and endogenous Usp4 accumulate in the nucleus. Further, using a Rev-green fluorescent protein-based export assay, we confirmed the existence of a nuclear export signal (133VEVYELKEL142) in Usp4. In addition, a functional nuclear import signal (766QPQKKKK772) was also identified, which was specifically recognized by importin α/β. Finally, we show that the equilibrium of Usp4 subcellular localization varies between different cell types. Usp4 is thus the first DUB reported to have nucleocytoplasmic shuttling properties. The implications of this shuttling for its function as a DUB are discussed.

Ubiquitin is a 76-amino acid protein that serves as a multifunctional covalent “tag” for a wide variety of cellular proteins. Its best understood function is in marking proteins for degradation by the 26 S proteasome, but ubiquitin conjugation is also important in diverse pathways including DNA repair, receptor internalization, and signal transduction (1, 2). Mutations affecting enzymes involved in the ubiquitin system have been implicated in several human diseases (1, 3, 4). Ubiquitin conjugation requires the action of several enzymes to activate and covalently attach ubiquitin to target proteins, with specificity being provided by the ubiquitin ligase/E3 enzymes, which are involved in substrate binding and have received much attention in recent years (5). Conversely, deubiquitylating enzymes (DUBs), which can cleave branched ubiquitin conjugates and/or linear ubiquitin precursor proteins, have received much less attention.

DUBs constitute large gene families in all eukaryotes and can be divided into the ubiquitin-specific protease (UBP) and ubiquitin C-terminal hydrolase families based on amino acid sequence (6–8). This multiplicity is not required merely for the cleavage of ubiquitin precursors but is indicative of other regulatory roles in the ubiquitin pathway. Clues to the function of some mammalian UBPs have come from their identification as growth regulators. These include the human tre-2/tre-17 (USP6) proto-oncogene (9) and the mouse cytokine-inducible UBPs DUB-1 and DUB-2 (10). We have studied the mouse Unp gene (Usp4 in the systematic nomenclature) (11), which was originally identified as a proto-oncogene related to tre-2/tre-17/USP6 (12, 13). Unp/Usp4 was subsequently observed to contain the UBP Cys and His boxes (9, 14) and since shown to have DUB activity (15, 16). In a study of primary human lung tumor tissue, Gray et al. (14) observed that the human homolog of Usp4, USP4 (UNP; also termed Unph), had consistently elevated gene expression levels in small cell tumors and adenocarcinomas of the lung, suggesting a possible causative role for this UBP in neoplasia. In a separate study using cell lines rather than primary tissue, USP4 protein levels were shown to be slightly but consistently reduced in cell lines derived from small cell tumors, leading to the suggestion that USP4 may be a candidate tumor suppressor gene (17). Recently, both mouse and human USP4 have been shown to interact with the Rb family of tumor suppressor proteins, a possible mechanism for USP4-mediated cell transformation (18, 19).

There is considerable controversy regarding the cellular localization of USp4/USP4. Initial reports described it as a nuclear protein as determined by cell fractionation (13). In contrast, cell fractionation studies by Frederick et al. (17) using different anti-peptide antibody showed it to be cytoplasmic. Both of these studies are clouded by the subsequent identification of a novel DUB termed USP15 (11, 20), which is 61% identical to USP4. Since the antibody produced by Frederick et al. (17) was raised against a peptide that is almost identical in the subsequence identified USP15, it seems likely that both USP4 and USP15 were being detected. Both USP4 and USP15 contain putative nuclear localization signals (NLSs), with that of USP4

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1 The abbreviations used are: DUB, deubiquitylating enzyme; CLSM, confocal laser-scanning microscopy; GFP, green fluorescent protein; GST, glutathione S-transferase; IMP, importin; LMB, leptomycin B; NES, nuclear export signal; pNLS, putative leucine-rich NES; NLS, nuclear localization signal; pNLS, putative NLS; PVDF, polyvinylidene fluoride; RFP, red fluorescent protein; UBP, ubiquitin-specific protease; FBS, fetal bovine serum; MPEF, mouse primary embryonic fibroblast; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay; T-ag, T-antigen.
it is being very similar to that of p53 (11, 13). Overexpression of transfectcd mvc epitope-tagged USP4 in IMR90 cells revealed either whole-cell or cytoplasmic staining (17). However, the overexpression of USP4 in these experiments could affect its location. Although the location of USP15 has not been studied, the recently described rat ortholog of USP15, UBP109 (21), shows both nuclear and cytoplasmic localization. Mutation of one of three putative NLSs in UBP109 resulted in exclusion from the nucleus of mvc-tagged UBP109 (21), although no quantification was presented.

Proteins larger than 40 kDa are generally transported into the nucleus through an active mechanism (22). Nuclear import is mediated by members of the importin (IMP) superfamily that recognize NLSs. Conversely, nuclear export is mediated by exportins that recognize NESs. Conversely, nuclear export is mediated by exportins that recognize NESs. The best studied exportins that recognize nuclear export signals (NESs), which are generally leucine-rich elements (23). The recently described rat ortholog of USP15, UBP109 (21), resulted in exclusion from the nucleus of mvc-tagged UBP109 (21), although no quantification was presented.

It seems reasonable to suggest that the conflicting reports of the cellular localization of USP4 reflect bona fide differences due to different cell types or stages of the cell cycle, rather than artifactual differences due to different technical approaches. In an effort to resolve this issue and to gain a deeper understanding of their function, we generated polyclonal antibodies that discriminate between Usp4 and Usp15 and used these to study their subcellular localization in a variety of cell types. We also constructed a fusion of the red fluorescent protein (RFP) to Usp4 and used this to identify functional NLS and NES signals. Usp4 is thus shown to be a nucleocytoplasmic shuttling protein, with different proportions of nuclear and cytoplasmic localization depending on the cell type. This is the first demonstration of nucleocytoplasmic shuttling for a DUB.

MATERIALS AND METHODS

DNA Manipulation and Cloning—DNA was amplified by PCR using Promega Pfu enzyme according to the manufacturer’s instructions. PCR templates were a mouse Usp4 cDNA clone (pCG53) (15) and a mouse Usp15 cDNA clone (pXX122) (20). GST fusion proteins were constructed by cloning the Usp4 or Usp15 gene region, corresponding to amino acids 539–789 or 526–797, respectively, into pGEX-4T-1 (Amer sham Biotech) using EcoRI and Sall sites. Poly-His-tagged full-length Usp4 and Usp15 proteins were constructed using the Gateway cloning system and the pDEST17 vector (Invitrogen). The RFP-Usp4 fusion was also cloned to the Gateway system in the plasmid pDEST-RFP, which was generated by inserting the attR1-cdAb-attR2 cassette B (Invitrogen) into the unique Smal site of plasmid pDsRed-C1 (Clontech) to render it Gateway-compatible.

The Rev-GFP reporter construct, Rev(L4)-GFP, was kindly provided by Beric Henderson (25, 26). Double-stranded synthetic oligonucleotides encoding putative Usp4 NLSs were ligated between the BamHI and AgeI sites of this vector. The Usp4-NLS2-β-galactosidase construct was created by introducing short double-stranded oligonucleotides encoding Usp4 NLS2 into XmaI sites of pPR2all vector (27). Site-directed mutagenesis was done using the QuickChange mutagenesis kit (Strat agene). Plasmids were purified for transfection using the PureFection maxi/midi kit (Promega). The integrity of all amplified DNAs and plasmids was confirmed by DNA sequencing (ABI Big Dye; Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University). Primer sequences are available on request.

Protein Purification and Antibody Production—E. coli strain BL21(DE3) was used for expression of Usp4 and Usp15-GST and His-tagged proteins, whereas MC1060 was used to express β-galacto sidase fusion proteins. β-Galactosidase fusion proteins were purified by affinity chromatography on the Molecular BioProducts (Rockford, IL) plasmid as described (27). Mouse IMP α2 (Rch1) and β1 proteins were expressed as GST fusion proteins in strain TG-1 and purified as previously (28). In the case of the Usp4 and Usp15 proteins, GST fusions were expressed in a 10-liter batch culture at 25 °C and induced with 0.01 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h and then purified by GSH affinity chromatography as described (29). His6-tagged fusions were expressed at 30 °C with 4 h of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside induction and were purified by affinity chromatography on nickel- iminodiacetate-Sepharose prepared as described (30). The GST-var iable region fusions were used to separately inoculate New Zealand White male rabbits (three injections of 150 μg) and produced by standard methods (31). The serum obtained was then affinity-purified using His6-tagged full-length Usp4 or Usp15 proteins immobilized on PVDF membrane by standard methods (31).

Cell Culture, Transfection, and Immunofluorescence—HeLa cells were grown in minimal essential medium, 10% fetal bovine serum (FBS), 2 mM t-glutamine. Saos-2 and SR-40 cells were grown in Mac Coys 5a, 15% FBS, 2 mM t-glutamine, 1 μM sodium pyruvate, and PSN (50 units of penicillin, 50 ng of streptomycin, 0.1 μg of neomycin per ml). Mouse primary embryonic fibroblasts (MPEFs) were grown in Dulbecco’s modified Eagle’s medium, 15% FBS, 2 mM t-glutamine. HepG2 cells were grown in minimal essential medium, 20% FBS, 1 mM amino acids, 1% nonessential amino acids, 1% vitamins. NIH3T3 cells were grown in Dulbecco’s modified Eagle’s medium, 10% FBS, 2 mM t-glutamine. MDA-MB-231 cells were grown in RPMI, 10% FBS, 2 mM t-glutamine, PSN. All cells were grown at 37 °C in 5% CO2 and passaged routinely. For immunofluorescence or transient transfection with RFP or GFP fusion constructs, cells were seeded onto glass coverslips in 12-well plates and transfected at 60–85% confluence with 2 μg of DNA and 4 μl of Cytofectene (Invitrogen) per 1 ml of medium as described by the manufacturer. Cells, expressing either RFP or GFP fusion proteins, were harvested at 48% paraformaldehyde for 15–30 min, washed, and mounted on slides for protein expression analysis 48 or 24 h posttransfection, respectively. For immunofluorescence, fixed and washed cells were permeabilized in PBS, 1% BSA, 0.1% SDS for 15 min, blocked in PBS plus 1% BSA for 45 min, incubated with primary antibodies (1.5 μg to 1:200 dilution in PBS plus 1% BSA) overnight at 4 °C, washed five times with PBS, incubated with anti-rabbit fluorescein isothiocyanate-conjugated or anti-mouse Texas Red-conjugated secondary antibody (1:100 dilution; Jackson laboratory) for 1 h, washed with PBS five times, and mounted on microscope slides using nail polish.

Image Analysis—Cells were imaged by confocal laser-scanning microscopy (CLSM; Bio-Rad Radiance 2000), using a × 60 oil immersion lens. The ratio of fluorescence in the nucleus relative to the cytoplasm (Fc/Fb) was derived by image analysis of confocal files as previously (28) using the ImageJ Image 1.62 public domain software, where the mean pixel density was assessed for manually cropped areas of uniform fluorescence in the nucleus (Fn) or cytoplasm (Fb), with identical measurements performed on untransfected cells used as the background fluorescence (Fb). The Fc/Fb ratio was calculated using the formula, Fc/Fb = (Fn − Pb)/(Fc − Fb), where an Fc/Fb of 0.9–1.1 was designated as N > C, Fc/Fb > 1.1 was designated as N > C, and Fc/Fb < 0.9 was designated as C > N. Statistical differences were determined by Student’s t test.

Western Blotting—Proteins were mixed with protein loading buffer (32), boiled for 5 min, and resolved by 10% Tris-glycine SDS-PAGE. Proteins were transferred to a PVDF membrane, which was then blocked in 5% skim milk, phosphate-buffered saline, 0.05% Tween 20 for 1 h, incubated with primary antibodies (1:1,000 dilution) for 1 h at room temperature, washed three times, incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (DAKO; 1:4,000 dilution) for 1 h at room temperature, washed, and visualized using the ECL detection reagent (Amer sham Biociences).

RESULTS

Generation of Usp4- and Usp15-Specific Antibodies—Several laboratories have previously generated anti-Usp4 antibodies, albeit with questionable specificity. For example, Gupta et al. (12, 13) generated anti-peptide antibodies, which reacted with a 180-kDa protein in NIH3T3 cell lysates instead of the predicted 110 kDa for Usp4. Frederick et al. (17) raised a peptide antibody against a region of Usp4 almost identical to the
Following several washes with PBS, cells were stained with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Jackson detection). The central two strips were stripped and reprobed with a mixture of Usp4 and Usp15 antibodies (lanes 4), and the membrane was reassembled prior to chemiluminescent detection. The position of the 97-kDa marker is shown. Usp4 expressed in bacteria gives proteins of different sizes due to internal initiation codons and/or degradation (15). D, western blot analysis with a commercial anti-USP4 antibody (Zymed Laboratories Inc.) followed by a horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:4,000) and ECL detection. The position of the 97-kDa marker is shown. Usp4 expressed in bacteria gives proteins of different sizes due to internal initiation codons and/or degradation (15). D and E, western blot against total mouse tissue extracts. Total protein extracts from mouse brain (Br), kidney (Ki), liver (Li), or spleen (Sp) were resolved by 8% SDS-PAGE, transferred to a PVDF membrane and probed with affinity-purified anti-USP4 antibody (D) or anti-Usp15 antibody (E). F, Usp4 and Usp15 antibodies detect proteins of different sizes. Mouse spleen extract was resolved in a wide slot gel by 6% SDS-PAGE and transferred to a PVDF membrane, and the membrane was sliced into four strips. Alternate strips were probed with affinity-purified anti-USP4 antibody (lanes 4) or anti-Usp15 antibody (lanes 15), and the membrane was reassembled prior to chemiluminescent detection. The central two strips were stripped and reprobed with a mixture of Usp4 and Usp15 antibodies (lanes 4 + 15). At higher magnification, two closely migrating bands can be detected in each lane of D–F, representing the alternately spliced variants. G–J, subcellular localization of endogenous Usp4 and Usp15 in HeLa and NIH3T3 cells. HeLa (G and H) and NIH3T3 cells (I and J) were fixed with 4% paraformaldehyde and stained with anti-Usp4 antibody (G and I) or with anti-Usp15 antibody (H and J) diluted 1:100 as described under “Materials and Methods.” Following several washes with PBS, cells were stained with anti-rabbit fluorescein isothiocyanate-conjugated secondary antibody (Jackson Laboratories), mounted on microscope slides, and analyzed by confocal microscopy.

In this light, we set out to generate specific antibodies to Usp4 and Usp15. The variable regions (var) of mouse Usp4 (amino acids 539–786) and mouse Usp15 (amino acids 526–797) (Fig. 1A) were chosen as antigens, since these regions were only 33% identical, compared with >60% for the remainder of the proteins. DNA encoding Usp4(var) or Usp15(var) was amplified by PCR and cloned into plasmid pGEX-4T-1. The resulting plasmids were used for bacterial expression of GST-Usp4(var) and GST-Usp15(var) fusion proteins, which were purified and then injected into rabbits for generation of antibodies.

Sera from immunized rabbits were affinity-puriﬁed using His$_6$-tagged full-length Usp4 or Usp15 and tested for their speciﬁcity by Western blotting. The antibodies recognized their cognate recombinant proteins and did not cross-react (Fig. 1C). These antibodies also detected recombinant human Usp4 or Usp15 speciﬁcally (data not shown). They also identiﬁed proteins of the expected molecular mass in extracts from various mouse tissues (Figs. 1, D and E). Splice variants of Usp4 have slightly lower molecular masses (108.3 or 103.7 kDa) than those of Usp15 (112.3 or 109.2 kDa) (20). A Western blot performed with spleen extract showed that the Usp4 antibody clearly detects a protein(s) of lower mass than the Usp15 antibody (Fig. 1F, lanes 4 compared with lanes 15). Upon closer examination, two closely migrating bands can be detected in each lane of D–F, representing the alternately spliced variants. In addition, a blot probed with a mixture of Usp4 and Usp15 antibodies detects thicker bands that encompass all bands seen with the separate antibodies (Fig. 1F, lanes 4 + 15). It was thus possible to conclude that our antibodies are speciﬁc for Usp4 and Usp15.

Differential Subcellular Localization of Endogenous Usp4 and Usp15—Usp4 and Usp15 are paralogs showing ~61% se-
sequence identity. This similarity is also reflected in the fact that both are ubiquitin-specific proteases and can bind the pRb family of tumor suppressor proteins (11, 18–20).

Immunostaining experiments showed that the subcellular localization of endogenous USP4 and USP15 proteins in HeLa human cervical cancer cells is largely mutually exclusive. Whereas USP4 was observed exclusively in the nucleus, excluding the nucleolus, USP15 was detected primarily in the cytoplasm and the nucleolus, but not the nucleus (Fig. 1, G and H). As a control, blocking either antibody with its cognate recombinant protein resulted in the elimination of staining (not shown), demonstrating the specificity of the antibodies and lack of cross-reactivity with other proteins in the fixed cell preparations.

These results were confirmed in the NIH3T3 mouse fibroblast cell line, although a greater heterogeneity of staining was observed. USP4 was found mainly in the nuclei of 23% of cells, exclusively in the cytoplasm of 27% of cells and throughout the cell in 50% of cells. USP15, in contrast, did not show such heterogeneity, being never seen in the nucleus but rather localized near the plasma membrane and enriched near some membranes but not others (Fig. 1, I and J).

Endogenous USP4 Has Different Subcellular Localization in Different Cell Lines—Since USP4 localization in HeLa and NIH3T3 cell lines was different, we explored the localization of endogenous USP4 in a range of other cell lines. Immunostaining of HepG2 (human hepatocellular carcinoma), Saos-2 (human osteosarcoma), and MDA-MB-231 (human breast cancer) cells (Fig. 2) revealed a range of different endogenous USP4 staining patterns. Thus, USP4 was detected only in the cytoplasm in HepG2 cells, while being exclusively nuclear in Saos-2 cells. Staining of MDA-MB-231 cells showed that USP4 was predominantly nuclear in 51.5% of cells, cytoplasmic in 27.5% of cells, and throughout the cell in 21% of cells (Fig. 2).

Such heterogeneity of subcellular localization of USP4 in a variety of cell lines implies that USP4 localization may be regulated in distinct fashion in different cell types. Pronounced nuclear localization of USP4 in HeLa and Saos-2 cells and nuclear and cytoplasmic localization in MDA-MB-231 and NIH3T3 cells implies that USP4 may contain special targeting sequences, such as NLS(s) or NES(s), mediating transport into and out of the nucleus. In order to test this hypothesis, we studied the effect of leptomycin B on the localization of endogenous USP4.

Endogenous USP4 Is Sensitive to Leptomycin B Treatment—Leptomycin B (LMB) is an antifungal agent that blocks nuclear export of proteins facilitated by the CRM1 nuclear export receptor, which recognizes Leu-rich NESs within the transported molecule (33, 34).

USP4 localization in response to LMB treatment was thus assessed in nontransformed MPEFs. In the absence of LMB, USP4 was mainly cytoplasmic (67% of cells) in MPEF cells, nuclear in 14% of cells, and throughout the cell in 19% of cells (Fig. 2). Treatment with 4 ng/ml LMB for 2, 4, 8, and 16 h caused gradual accumulation of USP4 in the nucleus from 14% at 0 h to 33% at 16 h (Fig. 3). The sensitivity of subcellular localization of endogenous USP4 to LMB indicated that USP4 contains an NES recognized by CRM1.

Exogenous USP4 Is Sensitive to LMB Treatment—In order to test sensitivity of exogenous USP4 to LMB, it was transiently expressed in HeLa cells as an RFP fusion protein. Expression of RFP-USP4 was analyzed at the single cell level by CLSM. Initial experiments in the absence of LMB revealed a disparate expression pattern. RFP-USP4 was observed either in the nucleus or cytoplasm or in both in the case of a small percentage of cells. This was in contrast to cells expressing RFP alone, which exhibited fluorescence throughout the cell, with no pronounced nuclear or cytoplasmic staining (Fig. 4).

Upon LMB treatment, cells expressing RFP-USP4 exhibited increased nuclear fluorescence compared with the untreated samples. RFP-USP4 localized in the nucleus of 85.5% of LMB-treated cells, compared with 49% in untreated cells. Determination of the nuclear to cytoplasmic ratio (Fnc/Fc) for LMB-treated samples indicated a value of 3.1 ± 0.3, significantly higher (p < 0.0001) than that in untreated cells (Fnc/Fc of 1.63 ± 0.13) (Fig. 4). LMB had no effect on the cells expressing RFP alone (Fnc/Fc of 1.03 ± 0.02, Fnc/Fc of 1.07 ± 0.03, p = 0.14, Fig. 4).

These results showed that exogenously expressed RFP-USP4 is similar to endogenous USP4 in terms of LMB sensitivity. Examination of the USP4 sequence revealed two putative leucine-rich NESs (pNESs). Additionally, two putative NLSs...
(pNLS), including that (pNLS2) described by Gupta et al. (13), were also identified (Fig. 1A).

**Usp4 Contains a Functional NES**—An in vivo export assay was used to assess the functionality of putative NESs of Usp4. The assay is based on the ability of a functional NES to export the HIV-1 Rev reporter construct, Rev(1.4)-GFP (25, 26), from the nucleus. The HIV-1 Rev protein contains strong NES and NLS sequences and is able to shuttle between the nucleus/ nucleolus and the cytoplasm of the host cell in order to export unspliced HIV mRNA. The reporter construct encodes a GFP fusion of Rev with a mutated (nonactive) NES and therefore accumulates in the nucleoli of transfected cells. Introduction of a functional NES into the Rev(1.4)-GFP reporter plasmid restores nuclear export of Rev and therefore can serve as a good indication of NES functionality.

Usp4 pNES1 and pNES2 were cloned into the Rev(1.4)-GFP reporter to generate Rev(1.4)-pNES1-GFP and Rev(1.4)-pNES2-GFP, respectively. Rev(1.4)-GFP was used as a negative control, and Rev(1.4)-wtRevNES-GFP, with reintroduced wild type RevNES, was used as a positive control (Fig. 5A).

The subcellular localization of all four transiently expressed GFP fusion proteins in HeLa cells was then compared. As expected, the negative control, Rev(1.4)-GFP fusion, was mainly localized in the nucleoli (89% of cells), with faint cytoplasmic staining observed in 11% of cells. The positive control, the Rev(1.4)-wtRevNES-GFP fusion, was localized exclusively in the cytoplasm of the majority of cells (80%) and throughout the cell in the rest (Fig. 5B). Rev(1.4)-pNES1-GFP fusion localized in almost identical fashion to the negative control, indicating that the pNES1 of Usp4 is not able to export the fusion protein from the nucleus in the presence of the Rev NES. In contrast, the Rev(1.4)-pNES2-GFP fusion revealed a strikingly different subcellular localization. Although all cells possessed some nuclear fluorescence, more than 72% of cells showed strong cytoplasmic fluorescence (Fig. 5B). Therefore, it can be concluded that Usp4 contains a functional NES (pNES2), which is weaker than the Rev NES, but is able to facilitate nuclear export of the Rev(1.4)-GFP reporter.

Several attempts to mutate Leu residues in either NES1 or NES2 in full-length RFP-Usp4 resulted in no detectable protein expression in transfected cells, suggesting that these regions are important for protein stability.

**Usp4 Contains a Functional NLS**—To investigate which of the Usp4 pNLSs were functional, each was mutated within the context of the pRFP-Usp4 expression plasmid, and a double mutant was also made (Fig. 6A). The mutant constructs were designated as RFP-Usp4NLS1, RFP-Usp4NLS2, and RFP-Usp4NLS1-2.

The RFP-Usp4NLS1 mutant did not show subcellular localization markedly different from that of RFP-Usp4 wild type in transfected HeLa cells. Quantitative analysis indicated no significant difference in the Fn/Fc ratios (Fn/FcRFP-Usp4wt = 2.03 ± 0.10, Fn/FcRFP-Usp4NLS1mut = 1.89 ± 0.09, p = 0.31) (Fig. 6B). This result implies that pNLS1 either does not act as a functional NLS or is totally redundant with another NLS in Usp4.

In contrast, the mutation of pNLS2 caused a significant change in Usp4 localization, whereby 25.4% of cells showed cytoplasmic localization of the mutant protein, compared with 1.7% in the case of the wild type protein. Consistent with this, the Fnc for cells expressing the NLS2 mutant was significantly lower than that for the wild type protein (p = 0.00007) (Fig. 6B), with a value of 1.45 ± 0.10. This result clearly indicates that pNLS2 is required for Usp4 nuclear localization.

Mutation of both pNLS1 and pNLS2 gave results for local-
matching bright field images are shown for each transfection. The schematic representation of the Rev(1.4)-GFP reporter shows where the Usp4 pNES1 or pNES2 or wild type RevNES were inserted between the Rev and GFP moieties (see "Materials and Methods" for details). The location of the NLS and mutated NES in Rev are inserted between the Rev and GFP moieties (see "Materials and Methods" for details). The location of the NLS and mutated NES in Rev are inserted between the Rev and GFP moieties.

A

B

C

Recombinant IMP α and β were expressed as GST fusion proteins (23). The amino acid sequence of NLS2 is shown by guest on July 24, 2018http://www.jbc.org/Downloaded from

functions of putative Usp4 NLSs. NLSs are shown in single-letter amino acid code with wild type residues shown on the left and mutated residues in boldface type. B: NLS2 is a functional NLS in Usp4. HeLa cells were transfected with the plasmids expressing RFP-Usp4 (wt), RFP-Usp4-NLS1, RFP-Usp4-NLS2, or RFP-Usp4-NLS1+2 mutants and observed by CLSM 48 h after transfection. Image analysis was performed as described in the legend to Fig. 4. C, effect of LMB on localization of RFP-Usp4-NLS2 mutant. HeLa cells were transfected with RFP-Usp4 (wt) or RFP-Usp4-NLS2mut constructs, and 48 h post-transfection, samples received 4 ng/ml LMB. 8 h after LMB addition, cells were imaged and then analyzed as described in the legend to Fig. 4.

Usp4-NLS2 Is Recognized by Importin α/β—The finding that NLS2 is functional prompted an attempt to elucidate the mechanism of NLS2-mediated nuclear import of Usp4. The first step of NLS-dependent nuclear protein import involves NLS recognition by IMPs (23). The amino acid sequence of NLS2 is similar to the basic NLSs, such as that of simian virus SV40 large tumor antigen (T-ag), which is recognized by the IMP α/β heterodimer. To test Usp4 recognition by IMPs, an ELISA-based binding assay was performed using two different Usp4 constructs: Usp4 NLS2 fused to β-galactosidase (Usp4-NLS2-β-galactosidase) and the full-length His6-tagged Usp4 (His6-Usp4). The first construct was used to test NLS2 recognition by IMPs in isolation, and the second one was used to test IMP binding in the context of the whole Usp4 protein. A protein containing the T-ag NLS fused to β-galactosidase was used as a positive control, whereas β-galactosidase alone was used as a negative control. Recombinant IMP α and β were expressed as GST fusion proteins as previously (11). The results show that both NLS2-containing proteins have a higher affinity for the IMP α/β heterodimer than for IMP α or β alone (Fig. 7, A and B). Interestingly, the affinity of IMP α/β for both fusion proteins was similar (Kd (His6-Usp4) = 54 nM, and Kd (Usp4-NLS2-β-gal) = 45 nM) and not dissimilar to the published binding affinities for IMP α/β-recognized NLS-containing proteins such as pRb (28) and the Rel family transcription factor Dorsal (35). Usp4-NLS2-β-galactosidase was also recognized by IMP α alone (Kd = 46.5 nM), but full-length Usp4 bound IMP α only weakly (Kd = 346 nM). Both proteins were recognized by IMP β with low affinity (Kd (His6-Usp4) = 107 nM, and Kd (Usp4-NLS2-β-gal) = 153 nM) (Fig. 7). The maximum level of IMP α binding to Usp4-NLS2 was half that of the IMP α/β heterodimer, consistent with the fact that both IMP α and β contain GST moieties. Overall, the results of the ELISA binding assay indicate that the IMP α/β1 | Nuclear Shutting of the Unp/Usp4 Deubiquitylating Enzyme

FIG. 5. Subcellular localization of Rev(1.4)-GFP reporter in the presence of Usp4 putative NESSs. A, Rev(1.4)-GFP reporter-based constructs. The schematic representation of the Rev(1.4)-GFP reporter shows the presence of Usp4 putative NESs. NLSs are shown in single-letter amino acid code with wild type residues shown on the left and mutated residues in boldface type. B, NLS2 is a functional NLS in Usp4. HeLa cells were transfected with the plasmids expressing RFP-Usp4 (wt), RFP-Usp4-NLS1, RFP-Usp4-NLS2, or RFP-Usp4-NLS1+2 mutants and observed by CLSM 48 h after transfection. Image analysis was performed as described in the legend to Fig. 4. C, effect of LMB on localization of RFP-Usp4-NLS2 mutant. HeLa cells were transfected with RFP-Usp4 (wt) or RFP-Usp4-NLS2mut constructs, and 48 h post-transfection, samples received 4 ng/ml LMB. 8 h after LMB addition, cells were imaged and then analyzed as described in the legend to Fig. 4.

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heterodimer is likely to be the nuclear import receptor for Usp4.

Involvement of the pRb Protein in Endogenous Usp4 Localization—Since Usp4 was recently shown to interact with the pRb family (18, 19), we examined the possibility that pRb may be involved in determining Usp4 subcellular localization. Usp4 localization was compared in Saos-2 (pRb-defective) (Fig. 8A) and SR-40 cells (Saos-2 cells expressing functional pRb) (Fig. 8B). SR-40 cells showed no difference in endogenous Usp4 localization compared with Saos-2 cells, being predominantly nuclear in both cases. As a control for pRb expression, both cell lines (Saos-2 and SR-40) were stained with anti-pRb antibodies (Pharmingen), confirming the cytoplasmic location of pRb in Saos-2 cells and expression throughout the cell with some nuclear accumulation in SR-40 cells (Fig. 8, C and D, respectively). Thus, at least in Saos-2 cells, expression of wild type pRb did not appear to modulate Usp4 subcellular localization.

DISCUSSION

The aim of this work was to investigate the cellular localization of Usp4 and resolve the issue of whether it is nuclear or cytoplasmic. Clearly, this information is important in determining the function of Usp4. We show for the first time that Usp4 is a shuttling protein containing functional targeting signals for nuclear import and export, which are recognized by distinct members of the importin family of nuclear import and export receptors. The Usp4 NLS,766QPQKKKK772, mutation of which drastically reduces Usp4 nuclear localization even when export is blocked by LMB, is recognized specifically by IMP/IMPβ or IMPγ as shown using ELISA-based protein binding assays. The Usp4 NES,133VEVYLLLKL142, was able to export a Rev(1.4)-GFP reporter protein from the nucleus. Although the export activity of Usp4 NES appeared to be low, the NES activity of some other cellular shuttling proteins, such as p53, oncoproteins hdm2 and PML, transcription factors AP2 and TFIIIA, and the transcription inhibitor protein IkBα, was even lower than that of Usp4 when tested in the identical export assay (25). This can be explained by the fact that the NESs of cellular proteins, including Usp4, were competing against the strong Rev NLS. The sensitivity of Usp4 to LMB indicates that CRM1 is the Usp4 nuclear export receptor. Thus, the apparent ability of Usp4 to shuttle between nucleus and the cytoplasm would appear to be in part due to the action of its nuclear import/export receptors IMPαβ and CRM1.

Based on the results here, the discrepancy in the literature regarding the subcellular localization of Usp4 can be explained by the ability of Usp4 to shuttle in signal-dependent fashion between nucleus and cytoplasm. Our immunostaining experiments showed that localization of endogenous Usp4 in a number of cell lines varies from exclusively cytoplasmic in HepG2 cells to exclusively nuclear in Saos-2 and HeLa cells. That transfected RFP-Usp4 fusion was seen in the cytoplasm as well as the nucleus in HeLa cells is almost certainly due to Usp4 overexpression and probable overload of the cellular nuclear transport machinery. The ostensibly disparate results of Gupta et al. (12) and Frederick et al. (17) are completely consistent with our results. Endogenous Usp4 was detected mainly in the nucleus of mouse NIH3T3 cells (12), whereas the overexpressed human ortholog of Usp4 was located mainly in the cytoplasm in IMR90 human lung fibroblasts (17). Thus, factors such as cell
type, stage of the cell cycle, or level of expression appear to influence the localization of a shuttling protein, changing the equilibrium to render it more nuclear or more cytoplasmic. That other shuttling proteins exhibit variable subcellular localization has been shown for the normally nuclear tumor suppressor p53, which is excluded from the nucleus in diverse cell types (36, 37) and in embryonic stem cells (38). Dostie et al. (39) have shown that subcellular localization of overexpressed eukaryotic translation initiation factor 4E depends on the level of overexpression. Clearly, localization of a shuttling protein such as Usp4 may differ between distinct cell types and under different conditions.

The mutation of NLS2 of Usp4 did not completely abolish its import into the nucleus. Similarly, LMB treatment of cells expressing either endogenous or exogenous Usp4 did not completely abolish Usp4 export from the nucleus. These findings imply that Usp4 may have alternative nucleocytoplasmic transport pathways, which do not involve classical positively charged NLSs or LMB-sensitive export receptors, like CRM1. Another possibility is that Usp4 intracellular transport is modulated through interaction with proteins such as those of the pRB family. The experiments with endogenous Usp4 (Fig. 8) showed that pRB is unlikely to be involved in Usp4 subcellular localization. We have preliminary data showing that the nuclear localization of Usp4 is increased in NIH3T3 cells expressing HPV E7 protein (data not shown), which targets p130 and p107 for proteasome-dependent degradation. Thus, p130/p107 may be involved in regulating Usp4 shuttling. Of significance in this context is the fact that p130 shuttles between nucleus and cytoplasm, with its nuclear export LMB-insensitive (40). In this context is the fact that p130 shuttles between nucleus and cytoplasm, with its nuclear export LMB-insensitive (40).

The antibodies raised during this work indicate that the subcellular localization of endogenous Usp4 and Usp15 is largely mutually exclusive. These results are significant, since thus far, it seems reasonable to postulate that nucleocytoplasmic shuttling of Usp4 is fundamental for its function. Substrates that are deubiquitylated by Usp4 in the nucleus/cytoplasm need to be identified in order to understand fully the cellular role of Usp4 and the apparent requirement for its shuttling activity.

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