The 19 S Proteasomal Subunit POH1 Contributes to the Regulation of c-Jun Ubiquitination, Stability, and Subcellular Localization*

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The AP1 (activator protein 1) transcription factor, c-Jun, is an important regulator of cell proliferation, differentiation, survival, and death. Its activity is regulated both at the level of transcription and post-translationally through phosphorylation, sumoylation, and targeted degradation. The degradation of c-Jun by the ubiquitin proteasome pathway has been well established. Here, we report that POH1, a subunit of the 19 S proteasome lid with a recently described deubiquitinase activity, is a regulator of c-Jun. Ectopic expression of POH1 in HEK293 cells decreased the level of c-Jun ubiquitination, leading to significant accumulation of the protein and a corresponding increase in AP1-mediated gene expression. The stabilization also correlated with a redistribution of c-Jun in the nucleus. These effects were reduced by mutation of a cysteine residue in the Mpr1 pad1 N-terminal plus motif of POH1 (Cys-120) and appeared to be selective for c-Jun, because POH1 had no effect on other proteasomal substrates. Our results identify a novel mechanism of c-Jun regulation in mammalian cells.

The 26 S proteasome is the principal site of controlled protein degradation in eukaryotic cells. The 2- to 2.5-MDa proteasome complex consists of a central core (20 S proteasome) and one or two multiprotein regulatory particles (RPs; 19 S proteasome), which are further organized into base and lid regions (1). The 19 S RP mediates the binding, deubiquitination, and unfolding of substrates into the 20 S core, where proteolysis takes place (2). One of the many cellular proteins targeted for degradation by the proteasome is the AP1 transcription factor, c-Jun (3). In addition to being the most potent transcriptional activator of its group, c-Jun is involved in a myriad of cellular activities, including tumorigenesis (4). Tight control of intracellular concentrations of active c-Jun is therefore required, a process that is achieved through rapid turnover by ubiquitination and degradation. Novel mechanisms for targeted ubiquitination of c-Jun were recently shown to be carried out by c-Jun-specific ubiquitin ligases, such as Itch (5) and SCF Fbw7 (6). Whereas much continues to be learned about mechanisms of c-Jun ubiquitination, little is known about the process of deubiquitination and its contribution to the overall regulation of c-Jun. Cellular deubiquitinases (DUBs) play an important role in controlled protein degradation (7); when bound to the proteasome they mediate the removal of ubiquitin chains as the substrate is being degraded, a step that facilitates substrate translocation into the 20 S chamber and allows for recycling of ubiquitin in the cell. DUBs also provide ubiquitin-editing activity, which can rescue particular substrates from being degraded by removing or trimming the degradation signal. A wide range of DUBs has been identified in eukaryotic cells, including enzymes with specificities for particular ubiquitinated substrates (8) but no specific c-Jun deubiquitinase activity has yet been reported.

A number of studies have implicated the 19 S proteasomal subunit, POH1 (also known as RPN11/pad1/S13/mpr1) as a possible regulator of AP1. Schizosaccharomyces pombe pad1/POH1 was shown to influence the activity of an AP1-like regulator, papi. Overexpression of pad1/POH1 in S. pombe led to enhanced AP1-dependent transcription of a downstream target gene without promoting papi mRNA expression (9). Reporter assays in mammalian HeLa cells also demonstrated that the overexpression of yeast pad1/POH1 potentiates c-Jun-mediated transcription (10). Subsequent studies showed that a flatworm orthologue of POH1, SmPOH1, selectively decreased degradation of c-Jun in vitro (11). More recently, POH1 was identified as an important DUB of the 19 S lid complex of the proteasome (12–14). Its activity within the complex contributes to substrate deubiquitination during proteasomal degradation and may also play a role in the editing of polyubiquitinated substrates as a means to control degradation (15). In addition, POH1 is believed to have other functions outside the proteasome, some of which do not seem to involve substrate deubiquitination (16–18). However, it remains unclear how these various activities relate to the stabilizing effect of POH1 on c-Jun. In this study, we investigate the mechanism of human POH1-induced c-Jun stabilization and explore its implications for the regulation of c-Jun activity in mammalian cells.

EXPERIMENTAL PROCEDURES

Constructs and Transfections—Human c-Jun, p27KIP, s5a/RPN10, and POH1 sequences were cloned by reverse transcription-PCR from HEK293 Total RNA and then confirmed by DNA sequencing. cDNA sequences were modified by PCR to introduce mutations and incorporate a FLAG, His, or HA tag, as indicated, and then subcloned into pCI-neo (Promega) or pTracer (Innogenetix). Plasmid pCMV-HA Ubiquitin was a gift from Dr. Bohmann’s laboratory (University of Rochester, Rochester, NY). POH1 Cys-120 mutants and deletion mutants were generated by PCR and subcloned into pCI-neo. All constructs were confirmed by DNA sequencing. For transient transfection of HEK293 cells, we used FuGENE 6 (Roche Applied Science), according to the manufacturer’s recommendations. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 20 mM Hepes. Unless otherwise specified, HEK293 cells were seeded in 6-well or 100-mm plates and were transfected with 1 or 4 μg
POH1 Regulates c-Jun Stability and Activity

of plasmid DNA, respectively. Total amounts of transfected DNA, in all experiments, were kept constant by addition of empty plasmids.

**Cell Fractionation and 20 S Proteasome Assays**—For glycerol gradient centrifugations, we prepared lysates from 5 × 10^7 HEK293 cells transfected with 4 μg of pCI-neo or pCI-neo.POH1-FLAG. Cleared cell lysates were layered on a 10–40% continuous glycerol gradient and subjected to a 22-h fractionation centrifugation at 25,000 RPM using an SW-28 rotor as previously reported (19). Samples were collected from the bottom of the centrifugation tubes, subjected to trichloroacetic acid precipitation, and aliquots of precipitated proteins were analyzed by SDS-PAGE and fluorography (20). 20 S proteasome assays were performed, according to standard procedures, using 0.5 μg of protein per reaction and Succ-LLVY-AMC (Biomol) as a degradation substrate. Reactions were carried out in a black 96-well plate, and fluorescence was recorded with the Fluostar Galaxy Fluorometer (BMG LabTechnologies) equipped with the appropriate excitation (λex = 380 nm) and emission (λem = 440 nm) filters.

**In Situ Stabilization, In Vitro Degradation, and Reporter Assays**—For the in situ stabilization assays, cells seeded in 100-mm plates were transfected with 0.6 μg of pTracer-c-Jun or pTracer alone, and 1.7 μg or 3.4 μg of pCI-neo.POH1-FLAG, one of the Cys-120 mutant constructs or empty vector. For the in situ stabilization assay of p27kip1, cells seeded in 100-mm plates were transfected with 0.6 μg of pTracer-p27kip1-FLAG and 3.4 μg of pCI-neo.POH1-FLAG. The pTracer construct encodes GFP under the control of a distinct promoter (Invitrogen) and was used as a transfection control. 48 h after transfection, cells were harvested in lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 10 mM EGTA, 2 mM EDTA, 10 mM NaVO₄, 20 mM NaF, 0.5% Nonidet P-40, 1 mM glycerophosphate, and protease inhibitor mixture from Sigma). 5 μg of cell lysate was then analyzed by immunoblotting with the appropriate antibodies. GFP expression was used to monitor transfection efficiency by fluorescence microscopy and protein loading by immunoblotting with anti-GFP (Molecular Probes/Invitrogen). For the in vitro stabilization assays, experiments were carried out as reported previously (11). Briefly, recombinant proteins were generated using 1 μg of each cDNA template in a coupled rabbit reticulocyte translation system (T7/SP6 TNT Reticulocyte System, Promega) in the presence or absence of [35S]methionine (Amersham Biosciences, 20 μCi/μl). Protein concentration was determined by labeling proteins with [35S]methionine and subjecting them to autoradiography followed by densitometry analysis. Reactions were terminated by addition of 50 μg/ml cycloheximide. The 35S-labeled c-Jun, p27kip1, or GFP proteins were incubated with an equimolar amount of in vitro translated unlabeled POH1 or a mutant POH1 protein in degradation buffer (20 mM Tris HCl, 1 mM dithiothreitol, 5 mM MgCl₂, 2 mM ATP (Sigma), 15 mM creatine phosphate (Sigma), and 2 units of creatine phosphokinase (Sigma)). 10-μl samples were removed at t = 0, 90, and 180 min and analyzed by gel autoradiography. For AP1 reporter assays, cells seeded in 6-well plates were transfected with 1 μg of pAP1-Luc (Clontech/BD Biosciences), 0.25 μg of pTracer-c-Jun, and 0.75 μg of pCI-neo.POH1, a Cys-120 POH1 mutant construct, or empty plasmid. The luciferase assays were later carried out using a luciferase assay kit (Promega) according to the manufacturer’s recommendations, and luminescence was recorded using a Fluostar Galaxy Luminometer (BMG LabTechnologies).

**Reverse Transcription and Quantitative Real-time PCR**—Total RNA from HEK293 cells transfected with a POH1 construct or vector (pCI-neo) alone was prepared using the RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions. Reverse transcription was performed on 1 μg of total RNA in a reaction volume of 20 μl using Superscript II Reverse Transcriptase (Invitrogen). Bcl1/Cyclin D1, BIM, Bcl3, CREB, and β-tubulin primers were designed using the Oligo Software (Molecular Biology Insights) and specificity was confirmed by BLAST data base analysis (see Table 1 for primer sequences). All oligonucleotides were obtained from Operon (Huntsville, AL) and reconstituted in RNase-free water (Invitrogen). Preliminary validation experiments demonstrated that the amplification efficiencies of the target genes and the internal reference (β-tubulin) were approximately equal. PCR reactions were carried out with the QuantiTect SYBR Green PCR kit (Qiagen) in a final volume of 10 μl using the Rotor-Gene RG3000 Instrument (Corbett Research). Cycling conditions were as follows: 95 °C for 15 min followed by 50 cycles of 95 °C for 15 s, 57 °C for 15 s, and 72 °C for 20 s. The generation of specific PCR products was confirmed by melting curve analysis and agarose gel electrophoresis. Quantitation of relative differences in expression levels were finally calculated using the comparative Ct method (20).

**In Situ Ubiquitination Assays**—For the in situ c-Jun ubiquitination assays, cells seeded in 6-well plates were transfected with 0.5 μg of pCI-neo.c-Jun-His, 0.5 μg of pCMV.HA-Ub, and 1 μg of pCI-neo.POH1, POH1C120S, POH1C120A, or empty plasmid and for the in situ p27kip1 ubiquitination assays, with 0.5 μg of pCI-neo.p27kip1-FLAG-His, 0.5 μg of pCMV.HA-Ub, and 1 μg of pCI-neo.POH1 or empty plasmid. 48 h later, cells were lysed directly into denaturing buffer (20 mM Tris Cl, 0.5 M NaCl, 5 mM imidazole, and 6 μM urea) and subjected to three freeze-thaw cycles. Ni²⁺-charged agarose beads (Novagen) were then added to the lysates for 2 h at 4 °C and then extensively washed with a 5 mM imidazole buffer followed by a 40 mM imidazole solution. Bound proteins were eluted by addition of reducing sample buffer containing 1 mM imidazole and finally analyzed by immunoblotting with anti-HA (Sigma) and anti-c-Jun (Oncogene/EMD Biosciences) or anti-p27kip1 (Cell Signaling Technology) antibodies. To assay for the effect of over-expression of POH1 on total protein ubiquitination and proteasome activity, cells seeded in 100-mm plates were transfected with 4 μg of pTracer or pTracer.POHa1 and analyzed by FACS using FACS Aria (BD Biosciences) equipped with an argon (λ = 488 nm) laser. 2 × 10⁵ sorted cells were seeded in 6-well plates and left overnight at 37 °C to recover. An equal number of non-transfected or transfected but unsorted cells was seeded as controls. 12 h later, cells were washed briefly with phosphate-buffered saline, scraped into 500 μl of phosphate-buffered saline, and lysed mechanically using a Dounce homogenizer. Protein concentrations were determined, and aliquots containing 5 μg of protein were analyzed by immunoblotting with anti-ubiquitin (Chemicon). An equal amount of protein was also assayed for 20 S proteasome activity using Succ-LLVY-AMC substrate, as described above.

**Immunofluorescence Assays**—For the immunofluorescence assays, cells seeded in 6-well plates were typically transfected with a total amount of 1 μg of plasmid/well. 48 h following transfection, cells were cooled on ice for 10 min, washed with phosphate-buffered saline, and later fixed and permeabilized with ice-cold methanol at −20 °C for 10 min. Cells were then washed and incubated with primary antibodies for 1 h at 4 °C. The incubation was followed by multiple washes with phosphate-buffered saline, and cells were supplemented with the appropriate secondary antibodies coupled to rhodamine or fluorescein isothiocyanate and left at 4 °C for 1 h. 5 min before the end of this incubation, 1 μg/ml 4,6-diamidino-2-phenylindole was added to the secondary antibody solution as a nuclear stain. Finally, cells were washed and mounted on slides for visualization by confocal microscopy. All images were acquired using a Zeiss LSM 510 confocal microscope. Fluorescein isothiocyanate, rhodamine, and 4',6-diamidino-2-phenylindole signals were obtained by excitation with argon (λ = 488 nm), HeNe (λ = 543
Homology Modeling—A theoretical model of POH1 was obtained based on the available crystal structure of AfJAMM (1R5X) (21). We first obtained a crude alignment of human POH1 and the AfJAMM template using the Consensus server (22). The alignment was examined to ensure that the positions of seed residues corresponding to JAMM amino acids were conserved. Structures were then generated using the COMPOSER module in Sybyl 6.9 (Tripos). Restraints were imposed on the JAMM residues to prevent exaggerated structural changes in this region during energy minimization. The model energies were minimized using the Powel subroutine with the Kollman_All atom force field at 0.05 kcal/mol Å until convergence. The final structures were analyzed with the WHATIF server and Ramachandran plot analysis. The structures of AfJAMM and human POH1 were subsequently aligned and superimposed using the Dalilite server (root mean square deviation = 1.4 Å, www.ebi.ac.uk/DaliLite/), and cartoons were generated using Pymol (23) and Sybyl 6.9 (Tripos). To assess the effect of the Cys-120 mutations, JAMM-Cys-120 was replaced with Ser or Ala using the Biopolymer module of Sybyl 6.9, and the models were minimized again to convergence.

Other Methods—Immunoprecipitations were performed using the Protein-A IP kit from Roche Applied Science. Immunoblotting was carried out according to standard procedures using the indicated antibodies, and dilutions were prepared to the manufacturer’s recommendations or determined experimentally as required.

RESULTS

We had previously used an in vitro degradation assay to demonstrate that a Schistosoma mansoni orthologue of POH1 stabilizes c-Jun (11). Here, we repeated this analysis with human POH1 and carried out a deletion mutagenesis study to identify the minimum region responsible for the c-Jun-stabilizing effect. A sequence alignment of POH1 orthologues highlights the positions of previously described functional motifs that were targeted for the mutagenesis (Fig. 1A). The most important of these motifs is a divalent metal ion binding stretch of amino acids named MPN (Mpr1 pad1 N-terminal plus) (12) or JAMM (JAB1/MPN/Mov34) (13). This motif

![Image of JAMM-Cys120 of POH1 is highly conserved in eukaryotic orthologues and is required for the stabilization of c-Jun in vitro.](image)

**FIGURE 1.** JAMM-Cys120 of POH1 is highly conserved in eukaryotic orthologues and is required for the stabilization of c-Jun in vitro. A, domain organization and ClustalW alignment of a putative ubiquitin-binding domain (UBD), the JAMM motif, and a putative nuclear export signal of POH1 orthologues. The position of the Cys-120 of interest is marked by a large arrow. The small arrow identifies the frameshift mutation P276A that yields the mpr1-1 phenotype in yeast (18). B, a schematic diagram of wild-type POH1 and mutants generated in this study. The putative ubiquitin-binding domain (UBD) and the JAMM domain are shown in gray. C, in vitro degradation of c-Jun. In vitro translated [35S]labeled c-Jun was incubated up to 3 h with rabbit reticulocyte lysate (RRL) alone or equal amounts of unlabeled in vitro translated POH1 wild-type or mutant proteins. Samples were removed at 0, 90, or 180 min and boiled immediately after addition of 2× SDS-sample buffer to stop the reactions and subsequently analyzed by SDS-PAGE followed by autoradiography. D, in vitro degradation of p27Kip and GFP. Reactions were performed as above, except that [35S]-labeled p27Kip or [35S]-GFP was used in place of c-Jun. In vitro degradation was assessed over the course of 3 h in the presence of an equal amount of unlabeled in vitro translated wild-type POH1 or RRL as a control. Samples were analyzed by SDS-PAGE followed by autoradiography.
POH1 Regulates c-Jun Stability and Activity

(EX_H(S/T)HX_SXXD) is closely related to the active site of a novel class of Zn\(^{2+}\)-associated metalloproteases (21) and is principally responsible for the DUB activity of the protein. Embedded within the JAMM sequence is a Cys-box-like motif of unknown function. The relevant cysteine (Cys-120) of this motif is not believed to be essential for the DUB activity of POH1 (12, 14) but may be required for deubiquitination of specific subsets of proteins (24). The alignment also identified a short hydrophobic peptide (LALLKML) located near the N terminus that resembles the ubiquitin-binding motif of RPN10 (LALALR/L/V) (25). At the other end of the protein is a newly discovered C-terminal functional domain that has been implicated in some of the non-proteasomal effects of POH1 (17) and is thought to be unrelated to substrate deubiquitination. We also identified a putative nuclear export signal motif resembling that of signalosome subunit JAB1, which may bind nuclear export protein CRM1 (26).

We have generated POH1 deletion mutants that lack a large portion of the JAMM motif (POH1ΔJAMM: 105–126) or the predicted Ub-binding peptide (POH1Δ37–44), as well as a series of C-terminal truncations. In addition, to test the role of the Cys-box motif, we produced two single amino acid substitutions of Cys-120, POH1C120S and POH1C120A (Fig. 1B). Wild-type POH1 and mutants were subsequently tested for their effects on c-Jun stability, using in vitro translated proteins in a cell-free degradation assay that contained rabbit reticulocyte lysates as a source of proteasomes and ubiquitination machinery, as well as cycloheximide to prevent new protein synthesis (Fig. 1C). The results show that 35S-labeled c-Jun degraded rapidly in the control sample but was significantly stabilized by addition of wild-type POH1 or a mutant containing an intact JAMM motif. Deletion of up to 143 residues from the C-terminal end did not change the stabilizing effect of POH1 on 35S-c-Jun. Mutant POH1Δ37–44 also decreased degradation of 35S-c-Jun though to a lesser extent than the wild-type or C-terminal deletions. In contrast, samples treated with a ΔJAMM mutant degraded very rapidly, as did the single point substitutions of Cys-120. Surprisingly, the half-life of 35S-c-Jun treated with either the POH1C120S or POH1C120A mutant was essentially indistinguishable from that of the control sample, suggesting this one cysteine was critical for the stabilizing effect of POH1 on c-Jun. To test whether POH1 could stabilize other proteins we repeated the in vitro degradation assays with p27\(^{KIP}\) or GFP (Fig. 1D). p27\(^{KIP}\) is a cyclin-dependent kinase inhibitor that is ubiquitinated and degraded by the proteasome (27), and GFP is a relatively stable protein that is not affected by proteasomal degradation except as a ubiquitin fusion degradation protein (28). There was no apparent effect of POH1 on the degradation rate of either protein.

Next we expanded the study into a cell-based system and examined the stabilization effect of POH1 on c-Jun in HEK293 cells. Because the mutation of residue Cys-120 of the JAMM motif was sufficient to abrogate the stabilizing effect of POH1 in vitro, we focused on this one residue for further analysis in situ. HEK293 cells were co-transfected with a fixed amount of c-Jun-expressing plasmid and two different amounts of plasmids expressing POH1, POH1C120S, POH1C120A, or vector only. Cells were supplemented with a small amount of c-Jun to facilitate detection of the protein because the endogenous level was found to be low. The c-Jun construct also encoded GFP, which was used as a loading control, and the total amount of plasmid was kept constant by addition of empty vector, as needed. Lysates of each test sample were subsequently analyzed by Western blotting using antibodies against c-Jun, recombinant POH1 (anti-FLAG) and GFP (Fig. 2A). The results show that overexpression of wild-type POH1 caused a strong, dose-dependent increase in the cellular levels of c-Jun, as predicted from the in vitro degradation assays. The POH1 cysteine mutants expressed at about the same level as the wild-type, based on densitometry analysis of anti-FLAG immunoreactivity. However, the mutants produced a much weaker or virtually no stabilization of c-Jun, depending on the dosage. At lower plasmid concentrations, the mutants had a minimal effect on c-Jun levels compared with wild-type POH1-FLAG, POH1C120A, or POH1C120S as indicated. Lysates collected 48 h following transfection were analyzed by Western blotting with anti-c-Jun antibody, anti-FLAG to detect recombinant wild-type POH1, or mutants and anti-GFP as a lane loading control. Black arrowheads represent nonspecific bands. Anti-c-Jun immunoreactive bands were subjected to pixel densitometry analysisand are represented as the means ± S.E. of three separate experiments. B, HEK293 cells were co-transfected with p27\(^{KIP}\)-HA and POH1-FLAG or vector alone, and lysates were analyzed by Western blotting with anti-HA, anti-FLAG, and anti-GFP antibodies as above. C, AP1 reporter gene assays. HEK293 cells were co-transfected with pAP1-Luc, pTracer.c-Jun, or pTracer alone, and a POH1 expression vector as indicated, or empty vector (pcI-neo). Lysates were subsequently tested for reporter gene activity by addition of luciferase substrate mix. Luciferase activities are expressed in relative light units (RLU) and are the means ± S.D. of three separate experiments. Cell lysates were also analyzed by Western blotting with an anti-GFP antibody as a control for transfection efficiency (lower panel).

![FIGURE 2. POH1 stabilizes c-Jun in situ.](image)

A, HEK293 cells were transiently transfected with a fixed amount of pTracer.c-Jun and increasing amounts of POH1-FLAG, POH1C120A, or POH1C120S as indicated. Lysates collected 48 h following transfection were analyzed by Western blotting with anti-c-Jun antibody, anti-FLAG to detect recombinant wild-type POH1, or mutants and anti-GFP as a lane loading control. Black arrowheads represent nonspecific bands. Anti-c-Jun immunoreactive bands were subjected to pixel densitometry analysis and are represented as the means ± S.E. of three separate experiments. B, HEK293 cells were co-transfected with p27\(^{KIP}\)-HA and POH1-FLAG or vector alone, and lysates were analyzed by Western blotting with anti-HA, anti-FLAG, and anti-GFP antibodies as above. C, AP1 reporter gene assays. HEK293 cells were co-transfected with pAP1-Luc, pTracer.c-Jun, or pTracer alone, and a POH1 expression vector as indicated, or empty vector (pcI-neo). Lysates were subsequently tested for reporter gene activity by addition of luciferase substrate mix. Luciferase activities are expressed in relative light units (RLU) and are the means ± S.D. of three separate experiments. Cell lysates were also analyzed by Western blotting with an anti-GFP antibody as a control for transfection efficiency (lower panel).
TABLE 1
Regulation of expression of endogenous c-Jun driven genes in cells overexpressing POH1

| Gene       | Size of amplicon (bp) | Relative expression levels | N*  | p value |
|------------|-----------------------|---------------------------|-----|---------|
| Bcl1/Cyclin D1 | 128                   | 1.48 ± 0.13               | 6   | 0.05    |
| BIM        | 132                   | 1.34 ± 0.13               | 6   | 0.01    |
| Bcl3       | 108                   | 1.44 ± 0.16               | 6   | 0.01    |
| CREB       | 100                   | 1.10 ± 0.20               | 6   | 0.65 (NS) |

* N is the number of independent determinations, each done in triplicate.

** NS, not significant.

and another proteasomal substrate (CREB) were unchanged (data not shown), thus confirming that c-Jun was selectively stabilized in these cells. Subsequent studies showed that the POH1-induced accumulation of c-Jun correlated with an increase in AP1-transcriptional activity (Fig. 2C). Transfection with wild-type POH1 stimulated AP1-mediated expression of a reporter gene (luciferase) ~2.5-fold, whereas the POH1C120A and POH1C120S mutants had no significant effect compared with the mock-transfected control. To test if this increased AP1 activity can influence expression of endogenous AP1-driven genes, we monitored the effect of POH1 overexpression on mRNA levels of three known c-Jun driven genes, Bcl1/Cyclin D1, BIM, and Bcl3 (Table 1) by quantitative Real-time reverse transcription-PCR. As a control, we determined the expression levels of CREB, which is not known to be influenced by c-Jun. The results showed that transfection with POH1 caused a small but statistically significant increase of ~40% in the expression level of each test gene, Bcl1, BIM, and Bcl3 compared with pCI-neo-transfected controls, whereas CREB expression levels were not significantly changed.

An important question arising from these findings is whether the recombinant POH1 expressed in HEK293 cells was incorporated into...
POH1 Regulates c-Jun Stability and Activity

FIGURE 4. POH1 overexpression influences the ubiquitination state of c-Jun. A, HEK293 cells seeded in 6-well plates were transfected with 0.5 μg of c-Jun-His and 0.5 μg of Ub-HA (lane 4), in combination with different expression vectors (1 μg each) as indicated (lanes 1–3), with HA-Ub alone (lane 3), or vector alone as control (lane 6). Ni²⁺-chelated proteins were purified from the different transfection groups and subjected to immunoblotting with anti-HA antibodies. The lower panel shows immunoblotting with anti-c-Jun antibody. B, HEK293 cells were transfected with vector alone (lane 6), 0.5 μg of HA-Ub (lane 5), or 0.5 μg of HA-Ub and 0.5 μg of p27kip-His (lane 4), or similar to lane 4 in combination with 1 μg of FLAG-POH1 (lane 3). Another group of transfected cells were further subjected to treatment with the proteasome inhibitor MG132 (50 μM) 3 h prior to preparation of cell extracts (lanes 1 and 2). Ubiquitinated proteins were prepared and analyzed as above. p27kip protein levels were examined using anti-p27kip antibody. Black arrowheads represent nonspecific bands. C, cells transfected with pTracer or pTracer-POH1 were subjected to analysis by FACS, and 2 × 10⁶ cells of each group were automatically seeded into 6-well plates for overnight recovery. Cells were then lysed and analyzed by Western blotting with anti-ubiquitin antibody, anti-β-tubulin (lane loading control), and anti-GFP (transfection control) antibodies.

The cellular proteasome and how that might influence the integrity and function of the complex. Transfection with a POH1 or POH1-FLAG plasmid under standard conditions caused an approximate 2-fold increase in total POH1 protein, as determined from densitometry analyses of anti-POH1 immunoreactivity (Fig. 3A). The recombinant POH1 was found to co-immunoprecipitate with the proteasome, and the same was true for the two POH1 cysteine mutants, which co-immunoprecipitated with the proteasome to about the same extent as the wild-type (Fig. 3B). Subsequent glycerol density gradient centrifugation analysis (19) determined that ~90% of all detectable POH1-FLAG sedimented with the proteasome in the higher density fractions. Moreover, the distribution of the recombinant POH1 in these fractions was virtually identical to that of the native protein, suggesting they were incorporated into the same complex. The remaining POH1-FLAG was localized in the lower density end of the gradient, presumably as a soluble-free form. No endogenous POH1 could be detected in the soluble fraction (Fig. 3C). Other studies determined that expression of POH1-FLAG had no significant effect on total peptidase activity measured against the fluorogenic proteasomal substrate, Suc-LLVY-AMC (Fig. 3D). In addition, the subcellular localization of the 20 S proteasome appeared to be unchanged in the overexpressing cells, based on in situ immunofluorescence analysis and confocal microscopy (data not shown). Thus the incorporation of POH1-FLAG does not appear to cause gross disruption of proteasome function or integrity, a finding consistent with earlier studies in yeast (17). We considered the possibility that an excess of POH1 could produce a change in the cellular amount of proteasomal complex that might explain the effects on c-Jun. To test this possibility we compared the levels of proteasome-associated immunoreactivity in glycerol gradient fractions of mock transfected and overexpressing cells. Fractions were monitored with antibodies targeting the 19 S RP lid (anti-RPN12), 19 S RP base (anti-Rpt1), the 20 S proteasome (anti-20 S α1, -2, -3, -5, -6, and -7) or anti-β-tubulin as a loading control (Fig. 3E). We detected little difference between the two cell populations aside from an apparent increase in the level of 19 S lid particles, as determined by immunoblotting with anti-RPN12. Based on densitometry analysis of anti-RPNI2 immunoreactivity relative to the cumulative tubulin signal, we estimated the amount of 19 S lid complex was elevated ~2-fold in overexpressing cells compared with the controls. The 19 S lid is a dynamic body, consisting of at least 17 subunits that are present in stoichiometric amounts and are capable of associating and dissociating from the larger proteasome (1). The results suggest that an excess of POH1 may promote de novo assembly of lid particles, which in turn increases the total amount of complex.

The in vitro mutagenesis analysis mapped the stabilizing effect of POH1 roughly to the N-terminal MPN domain of the protein, a region that includes the JAMM and Cys-box motifs as well as a putative ubiquitin-binding peptide. This led us to postulate that the DUB activity of protein, which is centered in this region, was likely involved in the stabilization of c-Jun. Initial attempts to demonstrate DUB activity of recombinant POH1 in vitro were unsuccessful (data not shown). Other laboratories have also been unable to detect DUB activity of purified POH1 (13, 14), suggesting the protein requires interactions with other lid subunits to become active. Thus we examined the effects of POH1 on the state of c-Jun ubiquitination in an intact cell environment. HEK293 cells were co-transfected with c-Jun-His, HA-ubiquitin, and POH1, POH1C120S, POH1C120A, or empty vector. Ni²⁺-chelated c-Jun-His proteins were isolated and subsequently analyzed by Western blotting with anti-HA antibody to detect ubiquitinated species or anti-c-Jun antibody to monitor for total amount of c-Jun recovered. The results show that transfection with wild-type POH1 caused a marked decrease in the amount of polyubiquitinated c-Jun conjugates compared with the mock transfected cells (Fig. 4A). In contrast, cells overexpressing POH1C120A or POH1C120S exhibited about the same level of c-Jun ubiquitination as the control. This is consistent with the model that POH1 causes deubiquitination of c-Jun in situ, although we cannot rule out an indirect mode of action leading to diminished ubiquitination of the protein. That the mutants were inactive indicates that Cys-120 is required for this effect and suggests that the stabilization of c-Jun is linked to a decrease in the level of ubiquitinated species, because the mutants also had little effect on c-Jun stability. The overexpression of wild-type POH1 did not cause a generalized decrease in protein ubiquitination, consistent with previous reports (24). We could not detect an effect on p27kip, either in the presence or absence of the proteasomal inhibitor MG132 (Fig. 4B), and there was no evidence of
global changes in the turnover of polyubiquitinated substrates. Immunoblot analysis of whole cell lysates with anti-ubiquitin antibody produced similar patterns of immunoreactivity in POH1-overexpressing, mock transfected, and untransfected cells (Fig. 4C).

In addition to effects on ubiquitination and stability, the transfection with POH1 was shown to influence the subcellular distribution of c-Jun. HEK293 cells were transfected with wild-type POH1, one of the cysteine POH1 mutants or empty vector as a negative control. In addition, transfections were carried out with a different 19 S subunit (RPN10) to test for specificity of the POH1 effect. Because the level of endogenous c-Jun was too low for clear visualization, cells were routinely co-transfected with a minimal amount of a c-Jun-HA-expressing plasmid (0.25 µg/well/6-well plate) and were subsequently monitored with an anti-HA antibody. As shown in Fig. 5A, overexpression of POH1 had a strong, dose-dependent effect on the distribution of c-Jun-HA. In the absence of added test proteins, most c-Jun-HA was present in the form of aggregates that resembled nuclear inclusions, as previously described (30). However, as the level of recombinant POH1 increased, c-Jun-HA became dispersed throughout the nucleus with accumulation in the region of the nuclear periphery, where apparent co-localization with POH1 was observed in at least 50% of the cells examined. Western blot analyses of fractionated cell lysates failed to detect significant anti-HA immunoreactivity in the cytosolic fraction (results not shown) and therefore c-Jun appears to be retained in the nucleus even at the highest dosage of POH1. Interestingly, transfection with the same amount of POH1C120S or POH1C120A had no effect on the distribution of c-Jun (Fig. 5B). The mutants and wild-type POH1 exhibited a similar pattern of nuclear and cytosolic expression, with all three proteins being particularly enriched in the region of the nuclear periphery. However, there was no visible relocalization of c-Jun-HA in cells transfected with the cysteine mutants, suggesting the effect requires the presence of Cys-120 and is likely related to diminished ubiquitination and stabilization of c-Jun. The subcellular localization of p27KIP-HA was also inspected in cells transfected with POH1 and was found to be unchanged relative to the vector transfected control (Fig. 5C). In addition, we were unable to detect an effect following overexpression of 19 S lid subunit, RPN10 (Fig. 5D), and, therefore, the relocalization of c-Jun appears to be a specific response to POH1.

**DISCUSSION**

POH1 is a well characterized deubiquitinase of the 19 S lid particle and is required for a functional proteasome. Studies in yeast have shown that loss-of-function mutations in endogenous POH1 (Rpn11) result in significant disruption of protein degradation, accumulation of poly-
POH1 Regulates c-Jun Stability and Activity

ubiquitinated conjugates, and, eventually, cell death (12, 14). As a proteasomal DUB that removes the polyubiquitin chains from incoming substrates, POH1 is believed to function in a dual capacity: the removal of the ubiquitin tag can facilitate substrate translocation into the 20 S chamber and thus facilitate proteolysis (13, 14), or it can cause the protein to be released from the proteasome allowing it to escape degradation. It has been suggested that POH1 works as a proteasomal “proof-reading” device that determines the fate of incoming substrates as to whether they will be degraded or rescued (15). The data presented in this study suggests that POH1 plays a particularly important role in determining the fate of c-Jun. POH1 was shown to cause a dose-dependent stabilization of c-Jun both in vitro and in intact cells. The stabilizing effect required the presence of an intact JAMM motif, the region responsible for the DUB catalytic activity, and was associated with a decrease in ubiquitinated c-Jun conjugates, suggesting it was mediated by deubiquitination of the protein. These effects were most likely caused by POH1 that was incorporated into the proteasomal 19 S complex, because most (~90%) of the recombinant protein sedimented with the proteasome and in the same high density glycerol fractions as native POH1. That the purified protein appears to lack DUB activity (Refs. 13 and 14 and this study) also suggests that POH1 was acting within the context of a complex. A possible explanation for these results is that the excess POH1 triggered de novo assembly of lid particles with a corresponding increase in the level of lid-associated DUB activity. This is supported by the observation that transfection with POH1 caused about a 2-fold elevation in 19 S lid immunoreactivity compared with the controls. However, we cannot rule out a different mechanism, possibly involving unincorporated POH1, which was also present in the transfected cells. It is noteworthy that POH1 overexpression did not produce global changes in protein stability or ubiquitination that might suggest a generalized effect on proteasomal function. In particular, we could not detect a significant effect on the stability or state of ubiquitination of p27KIP. We were also unable to see an effect on the stability of another short-lived protein, CREB (data not shown), suggesting that POH1 was able to discriminate among different proteasomal substrates. These results are consistent with earlier observations that the S. mansoni orthologue of POH1 had little stabilizing activity toward p53 under conditions where c-Jun was markedly stabilized (11), and the recent finding that POH1 overexpression in HeLa cells leads to significant stabilization of c-Jun but not the transcription factor Oct-1 (31). Together, these results suggest that POH1 selectively rescued c-Jun from degradation, most likely by removing its polyubiquitin anchor. This is the first evidence of a mammalian DUB controlling the state of ubiquitination and stability of c-Jun.

C-Jun plays numerous roles in mammalian cells, notably in the control of cell-cycle and apoptotic pathways, and therefore any mechanism that increases c-Jun stability is predicted to have important physiological consequences. In this study, we determined that the enhanced stability of c-Jun correlated with increased expression of an AP1 reporter (luciferase) and up-regulation of several endogenous c-Jun-driven genes (Bcl1, BIM, and Bcl3) (29). Although the effects reported here are due to overexpression of POH1, we note that POH1 protein levels were increased only ~2-fold in the transfected cells, suggesting that even a relatively modest change in the endogenous level of this protein is sufficient to influence c-Jun stability. POH1 is variably expressed among different cell types and can be up-regulated in response to environmental factors. For example, POH1 expression was increased up to 23.7-fold in the sponge Geodia cydonium after exposure to toxins under controlled conditions or in naturally contaminated waters (32). POH1 was also shown to be elevated in cancer cells, notably ovarian carcinomas, melanomas, and leukemic cells (33), where it can be used as a marker for malignancy. It will be of interest to determine how such fluctuations in POH1 influence c-Jun stability and activity in these systems.

One particularly unexpected finding of this study was that POH1 overexpression also altered the subcellular distribution of c-Jun. Little is known about the normal localization of c-Jun in mammalian cells, in part because, under normal conditions, the levels of the protein are often too low for detection, and the distribution varies among different cell types. HEK293 cells transfected with a small amount of c-Jun-HA showed immunoreactivity throughout the nucleus as well as several discrete nuclear bodies, where c-Jun-HA was highly enriched. Increasing the cellular concentration of POH1 caused the disappearance of these nuclear bodies and a dispersal of c-Jun throughout the nucleus. In contrast, the two POH1 cysteine mutants had no obvious effect, suggesting the re-distribution was linked to changes in c-Jun ubiquitination and stability. There is growing evidence that compartmentalization of nuclear proteins plays an important role in controlling key functions, including gene expression (34), ubiquitination (35), and protein degradation (36). The nuclear distribution pattern of c-Jun in the control cells resembles that of overexpressed transcription factor c-Myc, which is thought to be sequestered to the nucleolus for ubiquitination (37) and degradation by the proteasome (38). In HeLa cells, similar c-Jun-enriched inclusions have been shown to contain aggregates of polyubiquitinated protein and to co-localize with 20 S proteasomes (30), suggesting these structures are sites of protein degradation. Our results support the model that c-Jun was deubiquitinated in the presence of excess POH1, which, in turn, rescued the protein from degradation compartments and caused the aggregates to disassemble.

POH1 differs from other cellular DUBs in that it carries a conserved JAMM motif and is a Zn2+-dependent isopeptidase. In contrast, the majority of DUBs, including several proteasome-associated enzymes such as UBP6/USP14, UCH37/p37, and Dova/UBP4 (15), are members
of the cysteine protease superfamily. POH1 has a conserved Cys-box motif, similar to that of cysteine proteases, but its importance was downplayed following reports that Cys-120 mutations had no apparent effect on yeast viability or the intrinsic DUB activity of the 19 S particle (13, 14). Although Cys-120 does not appear to be essential for substrate debiquitination, it may nonetheless contribute to POH1 activity in more subtle ways. A recent study has shown that a C120A mutation of POH1 produced specific defects related to DNA replication and apoptosis. It is tempting to suggest that Cys-120 may be involved in a mechanism similar to that of some cysteine-switch metalloproteinases, which also affect only a subset of cellular proteins. The role of Cys-120 is worthy of further investigation.

A multitude of evidence has demonstrated the importance of the proteasome in cellular pathways of both health and disease (40–42), and several proteasome components have been implicated in the control of key regulators within these pathways. A better understanding of the pleiotropic roles of such proteasome subunits, including POH1, may unveil novel mechanisms of protein regulation.

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