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Characterization of Human Constitutive Photomorphogenesis Protein 1, a RING Finger Ubiquitin Ligase That Interacts with Jun Transcription Factors and Modulates Their Transcriptional Activity*[S]

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RING finger proteins have been implicated in many fundamental cellular processes, including the control of gene expression. A key regulator of light-dependent development in *Arabidopsis thaliana* is the constitutive photomorphogenesis protein 1 (atCOP1), a RING finger protein that plays an essential role in translating light/dark signals into specific changes in gene transcription. atCOP1 binds the basic leucine zipper factor HY5 and suppresses its transcriptional activity through a yet undefined mechanism that results in HY5 degradation in response to darkness. Furthermore, the pleiotropic phenotype of atCOP1 mutants indicates that atCOP1 may be a central regulator of several transcriptional pathways. Here we report the cloning and characterization of the human orthologue of atCOP1. Human COP1 (huCOP1) distributes both to the cytoplasm and the nucleus of cells and shows a striking degree of sequence conservation with atCOP1, suggesting the possibility of a functional conservation as well. In co-immunoprecipitation assays huCOP1 specifically binds basic leucine zipper factors of the Jun family. As a functional consequence of this interaction, expression of huCOP1 in mammalian cells down-regulates c-Jun-dependent transcription and the expression of the AP-1 target genes, urokinase and matrix metalloproteinase 1. The RING domain of huCOP1 displays ubiquitin ligase activity in an autoubiquitination assay *in vitro*; however, suppression of AP-1-dependent transcription by huCOP1 occurs in the absence of changes in c-Jun protein levels, suggesting that this inhibitory effect is independent of c-Jun degradation. Our findings indicate that huCOP1 is a novel regulator of AP-1-dependent transcription sharing the important properties of *Arabidopsis* COP1 in the control of gene expression.

RING finger domains are found in a large number of proteins involved in many fundamental cellular processes, such as proliferation, apoptosis, development, oncogenesis, and viral pathogenicity (1–3). The RING domain is characterized by a conserved pattern of cysteine and histidine residues that bind two zinc atoms in a particular cross-brace arrangement. A unifying function for the RING has been difficult to define, given the large number of molecular pathways that are affected by RING proteins. It has been proposed that a general feature of RING domains is the ability to mediate interactions with other RING or non-RING proteins and to contribute to the assembly of macromolecular complexes (1). More recently, a role for RING domains in ubiquitination reactions has been identified, and several RING finger proteins have demonstrated ubiquitin ligase activity, with the ability to transfer ubiquitin to a substrate as well as to themselves (4–6).

Many RING finger proteins have been implicated in regulating transcription through a variety of mechanisms (7), including ubiquitination and degradation of positive (8–12) or negative regulators of transcription (13–15). Pathways independent of protein degradation have also been described and may involve protein sumoylation (16), changes in promoter recruitment (17), interference with components of the transcriptional machinery, such as general transcription factors (18, 19) or RNA polymerase II (20, 21), changes in histone acetylation (22, 23), or chromatin rearrangement (24, 25).

The *Arabidopsis thaliana* RING finger constitutive photomorphogenesis protein 1 (atCOP1),[1] has been characterized for the profound effect that loss of function mutations have on *Arabidopsis* photomorphogenic development (26). atCOP1 influences *Arabidopsis* growth by regulating the pattern of gene expression in seedlings. Work from X. W. Deng's laboratory (27) demonstrated that under defined extracellular conditions (darkness) atCOP1 accumulates in the nucleus, where it binds one or more transcriptional regulators, such as the basic leucine zipper (bZIP) factor HY5 (28). Degradation of HY5 in the dark is dependent on the presence of wild type at COP1 in the nucleus (29). In addition, COP1 seems to interact function-

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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[1] The abbreviations used are: atCOP1, *A. thaliana* constitutive photomorphogenesis protein 1; bZIP, basic leucine zipper; CSN, COP9 signalosome; huCOP1, human constitutive photomorphogenesis protein 1; NLS, nuclear localization sequence(s); HA, hemagglutinin; MMP1, matrix metalloproteinase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MoAb, monoclonal antibody; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GST, glutathione *S*-transferase.

ally with other proteins, because nuclear accumulation of atCOP1 requires the activity of several genes (26), many of which encode subunits of a single multimolecular complex, the COP9 signalosome (CSN) (30).

Our laboratory has been investigating the role of the mammalian CSN and its functional regulation in response to extracellular signals, such as intercellular adhesion (31). Because atCOP1 is integral to the function of the plant CSN, we sought to obtain further insight into the function of the mammalian CSN by studying the human COP1 orthologue. In this paper we report the cloning and characterization of human COP1 (huCOP1). Similar to its plant counterpart, huCOP1 contains seven WD40 repeats in the carboxyl terminus and an amino-terminal RING domain, which displays ubiquitin ligase activity in an autoubiquitination assay *in vitro*. We show that huCOP1 possesses a nuclear localization sequence (NLS) that directs distribution of the protein to the nucleus. In co-immunoprecipitation assays huCOP1 specifically binds transcription factors of the Jun family through a highly conserved amino acid motif. The functional consequence of this interaction is the down-regulation of c-Jun transcriptional activity by ectopically expressed huCOP1 protein. Our findings indicate that also in human cells COP1 may function as a regulator of gene expression, affecting the AP-1-dependent pathway, which plays a central role in mammalian cell proliferation and differentiation.

EXPERIMENTAL PROCEDURES

Cloning of huCOP1—Translated BLAST search of a human expressed sequence tag data bank revealed several expressed sequence tags with a high degree of identity with *A. thaliana* COP1. IMAGE clone 1707833 was obtained from Human Genome Mapping Project Resource Center (Cambridge, UK) and used to screen a phage library from NT2/D1 cells (Stratagene). A positive clone was amplified, and phage DNA was extracted. The cDNA insert (2.6 kb) was excised and sequenced. Primer extension experiments and 5′ rapid amplification of cDNA ends-PCR revealed that although huCOP1 cDNA extended further 5′ for 500 nucleotides, an in-frame stop codon was present 150 nucleotides upstream of the identified ATG (GenBank™ accession number: NP_071902). HeLa cell RNA was extracted with the RNAeasy kit (Qiagen) following the manufacturer's instructions. For primer extension assays, 50 μg of total RNA were precipitated overnight with 40 pmol of each of the following ^{32}P-labeled primers: COP1.16, 5′-GGCTACCAGACATCGTGACTCC-3′, and COP1.17, 5′-GGATAAAGACGAGGAGGCGGAAGTCACCGA-3′. Annealing occurred in hybridization buffer (150 mM KCl, 10 mM Tris, pH 7.5, 1 mM EDTA) at progressively decreasing temperatures (from 80 to 42 °C), retrotranscription was carried out with the Superscript II enzyme (Invitrogen) at 42 °C. After RNase treatment and phenol/chloroform extraction, the samples were precipitated and resolved on a 6% polyacrylamide sequencing gel. Amplification of 5′ cDNA ends was performed with the above primers and the GeneRacer kit (Invitrogen), following the manufacturer's protocol. Sequence homologies were determined with the ClustalW multiple alignment tool.

Northern Blot Analysis—huCOP1 cDNA was digested with *Eco*RI to yield a 900-nucleotide fragment. After gel purification, the probe was labeled with [^{32}P]dCTP using a *Redi*prime II kit (Amersham Biosciences) and hybridized to commercial Northern blot membranes (FirstChoice Northern Human Blot 1 and FirstChoice Northern Human Blot 2; Ambion) containing ~2 μg of poly(A) RNA from different human tissues. The membranes were stripped and subsequently probed with a β-actin cDNA probe.

Production of Monoclonal Antibodies and Western Blotting—The amino-terminal fragment of COP1 (amino acids 1–452) was cloned into pGEX4T1 (Pharmacia Corp.). Recombinant GST-COP1 was purified from *Escherichia coli* by binding to glutathione-Sepharose (Pharmacia Corp.) and used to raise monoclonal antibodies against COP1 (SP212). SP212 recognized overexpressed FLAG-COP1 by Western blotting and by indirect immunofluorescence. Lysates from transfected COS-7 cells and human cell lines were obtained by boiling samples for 5 min in 2% SDS, followed by the addition of RIPA correction buffer (32). Tissue homogenates from rat embryo (embryonic day 18.5) brains were obtained by mechanical lysis in hypotonic buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM Pefabloc SC; Roche Applied Science), cleared by centrifugation (13,000 rpm, 30 min) and filtration (0.3 micron), and analyzed by denaturing SDS-PAGE and Western blotting with SP212 or antibodies against β-tubulin (Sigma). Detection was performed with the ECL Plus reagent (Amersham Biosciences).

Indirect Immunofluorescence—Full-length COP1 or PCR amplifications of the indicated truncation products of COP1 (RINGcc, 1–344; RING, 1–214; ccWD40, 208–731; and WD40, 401–731) were cloned into the pFLAG-CMV2 vector. Mutations in the NLS of full-length COP1 and of RINGcc were introduced by site-directed mutagenesis using the PCR-based method QuikChange (Stratagene). Arg^{111} and Arg^{113} were both mutated to alanine. COS7 cells were grown on glass coverslips in 35-mm dishes and transfected using the FuGENE 6 reagent (Roche Applied Science). 0.2 μg of DNA/35-mm dish were used. 48 h after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Nonidet P-40, and incubated with anti-FLAG antibodies (M5; Sigma), followed by fluorescein isothiocyanate anti-mouse antiserum. The cells were analyzed with a Bio-Rad laser scanning confocal microscope MRC-1024.

Co-immunoprecipitations—COS7 cells in 60-mm dishes were transfected using FuGENE 6 and 0.6 μg of the indicated cDNAs. The lysates were harvested after 48 h in Nonidet P-40 lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, with protease inhibitors). Postnuclear supernatants were cleared by incubation with protein G-Sepharose (Amersham Biosciences) for 30 min at 4 °C and then incubated with anti-FLAG antibodies (M5) or anti-HA antibodies (12CA5) for 1 h at 4 °C, followed by the addition of protein G-Sepharose and further incubation for 20 min. Bound proteins were washed four times with lysis buffer and eluted with Laemmli sample buffer. The samples were loaded on 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with the indicated antibodies. Anti-c-Jun, anti-JunD, and anti-c-Fos antibodies were from Santa Cruz Biotechnology, Inc. Detection was performed with the ECL Plus system (Amersham Biosciences).

In Vitro Ubiquitination Assay—Mutations in the RING domain of full-length COP1 and of the RINGcc deletion mutant were introduced by site-directed mutagenesis using the PCR-based method QuikChange (Stratagene). Cysteine 456 and 459 were mutated to alanine. Full-length COP1, COP1 RINGcc (amino acids 1–412), and the respective RING mutants were subcloned into the pGEX4T1 vector (Pharmacia Corp.), expressed in *E. coli* BL21 (DE3) and purified from bacterial lysates with glutathione-Sepharose 4B beads (Amersham Biosciences). His-tagged ubiquitin (a gift from M. Pagano), engineered to contain a protein kinase recognition site (LRRASV), was purified with nickel beads and labeled with ^{32}P by incubating [γ-^{32}P]ATP and cAMP kinase (Sigma) at 37 °C for 30 min. *In vitro* ubiquitination assays were performed as described (4). Briefly, reaction mixtures containing 0.3 μg of GST fusion proteins bound to GSH-Sepharose were incubated for 1 h at 30 °C in the presence of His-ubiquitin labeled with ^{32}P and the purified enzymes: E1 (10 ng, affinity), His-tagged UbcH5B (300 ng) in ubiquitination buffer (25 mM Tris, pH 7.6, 5 mM $MgCl_2$, 0.1 M NaCl, 1 mM dithiothreitol, 2 mM ATP). The reactions were stopped with 5× SDS sample buffer, and the samples were resolved by SDS-PAGE. Ubiquitinated proteins were detected by autoradiography.

Reporter Gene Assays—293T cells were grown in 35-mm dishes and transfected in triplicate samples with 0.2 μg of an AP-1-driven luciferase reporter gene (33), 0.1 μg of a β-galactosidase reporter construct, 0.2 μg of RSV c-Jun, and the indicated COP1 constructs, using the FuGENE 6 reagent, following the manufacturer's protocol. After 24 h the cells were collected, and the luciferase activity was analyzed in cell lysates following the manufacturer's instructions (Promega). β-Galactosidase activity was measured in the same samples (Roche Applied Science). For the analysis of the urokinase promoter activity, 0.8 μg of luciferase reporter constructs containing either the Sp1 responsive proximal promoter (−86 to +32) or the proximal promoter and the AP-1 enhancer region (−1977/−1880) in the pGLBasic vector were used (34).

Real Time Fluorogenic Reverse Transcriptase-PCR—Total RNA was isolated from 35-mm dishes of transfected 293T or HeLa cells with the RNeasy mini kit (Qiagen). Transfection (0.2 μg of huCOP1 or empty vector, plus 0.3 μg of c-Jun, for u-PA detection or 0.3 μg of c-Jun and 0.3 μg of c-Fos) were conducted in triplicate. 1 μg of total RNA was reverse-transcribed with Superscript II Reverse Transcriptase, following the manufacturer's protocol (Invitrogen). Real time fluorogenic reverse transcriptase-PCR was performed according to manufacturer's instructions (PE Applied Biosystem). 10–40 ng cDNA were amplified in 25 μl of final volume in the presence of 1.25 μl of the following "Assays-on-Demand" oligonucleotides purchased from Applied Biosystems: uroki-

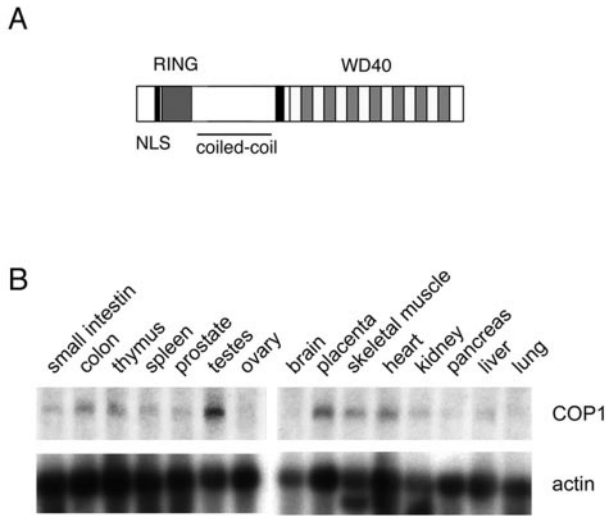

FIG. 1. **COP1 is highly conserved and it is ubiquitously expressed in human tissues.** *A*, prediction of COP1 protein domains, according to the Pfam data base, version 7.4. The *vertical black bars* represent predicted NLS. The carboxyl-terminal half of the protein contains seven WD40 repeats. A region between the amino-terminal RING domain and the WD40 repeats is predicted to assume a coiled-coil conformation. *B*, Northern blot analysis of huCOP1 expression in a panel of human tissues was carried out with a cDNA probe comprising the 5′ fragment of huCOP1 (*top panel*). Hybridization with an actin probe was used as a loading control (*bottom panel*).

nase plasminogen activator, tumor necrosis factor-α, matrix metalloproteinase 1 (MMP1), GAPDH, and 18 S. Relative mRNA levels (compared with mock transfected samples) were calculated according to the manufacturer's instructions and normalized to GAPDH mRNA levels. Similar results were obtained with normalization to 18 S RNA levels.

RESULTS

Cloning of Human COP1—To identify human homologues of the atCOP1 protein, we performed a similarity search of a human expressed sequence tag data base using a translated Blast search program (35). An expressed sequence tag clone whose conceptual translation showed 52% identity with atCOP1 was chosen to screen a human cDNA library and to isolate a 2.6-kb cDNA clone. Primer extension experiments indicated that the mRNA start site was located 600 nucleotides upstream of the first nucleotide of the isolated clone (not shown). Retrotranscription of HeLa mRNA together with 5′ rapid amplification of cDNA ends-PCR and sequencing of the amplified products allowed the identification of an in-frame STOP codon 153 bp upstream of the first ATG of the isolated clone (see GenBank™ accession number NP_071902), confirming that it comprised the full-length coding sequence. Sequencing of the isolated clone revealed a predicted 2193-nucleotide open reading frame, encoding a protein of 731 amino acids with a good degree of conservation with atCOP1 (34% identity, 64% overall similarity) (36). In particular, the analysis of huCOP1 protein domains demonstrated that all of the structural modules present in atCOP1 (26) are conserved in the human protein (Fig. 1*A*); in addition to seven WD40 repeats in the carboxyl-terminal domain (51% identity with atCOP1), preceded by a predicted coiled-coil structure, a zinc-binding RING motif is present in the amino-terminal portion of the protein. Two putative NLS could be identified: a monopartite motif located amino-terminal to the RING domain (amino acids 111–114) and a bipartite NLS proximal to the WD40 repeats. Most of the divergence between atCOP1 and huCOP1 is present in the amino-terminal part, which is more extended in the human protein.

Expression of COP1 in Mammalian Tissues—Northern blot analysis of COP1 transcripts showed widespread expression in normal human tissues. Highest levels were detected in testes, placenta, skeletal muscle, and heart (Fig. 1*B*).

A monoclonal antibody (MoAb) raised against recombinant huCOP1 recognized the FLAG-tagged version of the transfected protein in Western blot analysis (Fig. 2*A*, *left lane*), as well as an additional band with a slightly lower molecular weight, compatible with the untagged endogenous protein, present in lysates both from transfected COS7 and untransfected HeLa cells (Fig. 2*A*). Extensive Western blot analysis of COP1 expression in a panel of cell lines showed that endogenous levels of COP1 are in general low (or undetectable) in lysates derived from human cell lines and cultures (examples are shown in Fig. 2 (*A* and *B*, *right panel*)). The anti-COP1 MoAb also recognized the murine form of the protein (GenBank™ accession number NP036061; 97% identity with huCOP1) in lysates from embryonic rat brain and in the murine embryonic cell lines analyzed (Fig. 2*B*, *left panel*).

Structural Determinants of the Subcellular Localization of huCOP1—To study the role of the different protein domains in determining huCOP1 subcellular localization, we transfected FLAG-tagged versions of full-length huCOP1 and of various huCOP1 deletion constructs in COS7 cells and analyzed the distribution of the proteins by indirect immunofluorescence. Transient expression studies demonstrated that huCOP1 is present both in the cytoplasm and the nucleus (Fig. 2*C*), where it has a speckled distribution, with preferential enrichment at the nuclear periphery. Constructs lacking the amino-terminal domain (FLAG-ccWD40 and FLAG-WD40; Fig. 2*C*, *bottom row*) showed cytoplasmic localization, in contrast to mutants carrying carboxyl-terminal deletions (FLAG-RINGcc and FLAG-RING; Fig. 2*C*, *middle rows*), which are preferentially concentrated in the nucleus. This finding implied the existence of a signal for nuclear localization in the amino-terminal portion of the protein. Consistently, point mutations of Arg^{111} and Arg^{113} in the putative monopartite NLS are sufficient to prevent nuclear entry not only of the full-length protein but also of the FLAG-RINGcc construct, resulting in a complete relocalization of the protein (FLAG-COP1mNLS and FLAG-RINGccmNLS; Fig. 2*C*, *right panels*).[2]

Given the role of WD40 repeats in establishing protein-protein interactions (37), it is not surprising that huCOP1 mutants that lack these motifs showed a less localized pattern of nuclear distribution (Fig. 2*C*, *row 2*, *central panel*), indicating that the WD40 repeats may be necessary to retain huCOP1 in the correct subnuclear location. Further deletion of the coiled-coil region resulted in a complete diffused nuclear pattern (FLAG-RING; Fig. 2*C*, *row 3*, *central panel*), suggesting that this region may also be involved in protein-protein interactions.

In *Arabidopsis*, the coiled-coil domain of atCOP1 has been implicated in the homodimerization of the protein. We tested whether huCOP1 can also dimerize by transfecting HA-tagged full-length huCOP1 together with either FLAG-tagged full-length huCOP1 protein (FLAG-COP1) or one of several COP1 deletion constructs, as indicated (Fig. 3). Only constructs containing the coiled-coil region of the protein (FLAG-COP1, FLAG-RINGcc, and FLAG-ccWD40) were co-precipitated with HA full-length huCOP1 by anti-HA antibodies, confirming the importance of this domain for the self-dimerization of the human protein as well.

HuCOP1 Has Ubiquitin Ligase Activity in Vitro—In *Arabi-*

[2] During revision of this paper, a report from Yi *et al.* (Yi, C., Wang, H., Wei, N., and Deng, X. W. (2002) *BioMed Central Cell Biology*, 3(1):30, www.biomedcentral.com/1471-2121/3/30) has appeared, describing a subcellular distribution for the murine COP1, which is strongly consistent with our findings relating human COP1.

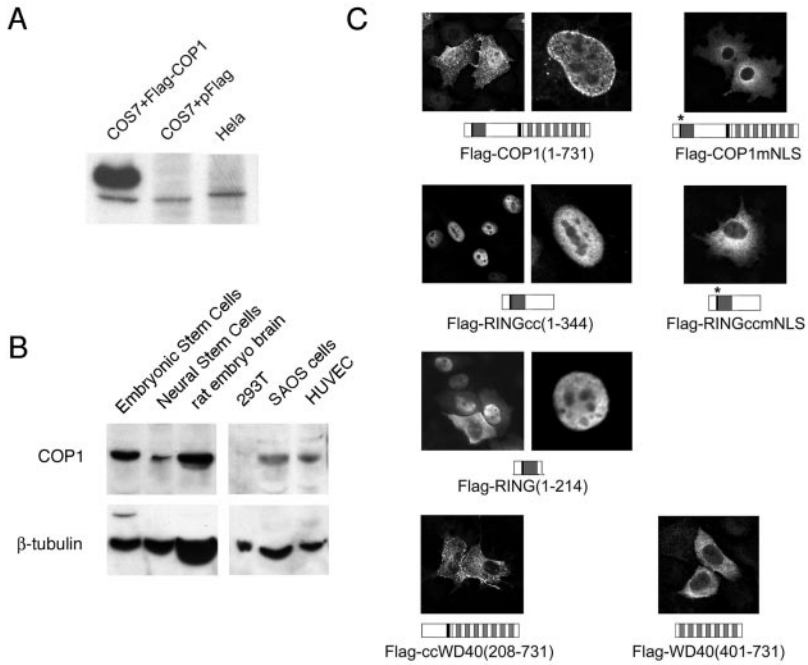

FIG. 2. **Mammalian COP1 is detectable in human and mammalian cells and localizes to both the nucleus and the cytoplasm.** *A*, anti-COP1 monoclonal antibody recognizes transfected and endogenous huCOP1. COS7 cells were transfected with 2 μg of FLAG-tagged full-length COP1 (*left lane*) or pCMVFLAG vector (*middle lane*). Total HeLa cell lysates (*right lane*) were extracted with 2% boiling SDS buffer. Total cell lysates were blotted with anti-COP1 antibodies raised against recombinant COP1. *B*, endogenous COP1 expression was analyzed by Western blotting with anti-COP1 antibodies of lysates from human (*right panels*) or murine (*left panel*) cell lines and tissues. Cellular content in lysates was estimated by blotting with anti-β-tubulin antibodies (*bottom panel*). *C*, epitope-tagged full-length COP1 (FLAG-COP1) or the indicated deletion constructs were transfected in COS7 cells. Distribution of the transfected proteins was analyzed by indirect immunofluorescence and confocal microscopy. The *middle panels* in *rows 1–3* show enlarged details of the nuclear distribution of the different constructs. *Right panels*, *rows 1* and *2* show expression patterns of full-length COP1 and of the RINGcc truncated construct carrying a mutation (*asterisk*) in the first predicted NLS. NLS mutants are exclusively localized to the cytoplasm.

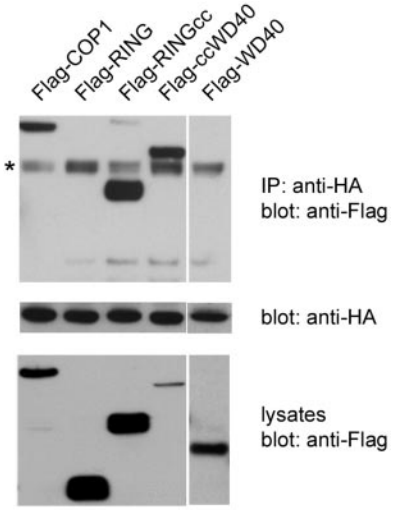

FIG. 3. **huCOP1 homodimerizes through its coiled-coil region.** COS7 cells were transfected with HA-tagged full-length huCOP1 and the indicated FLAG-tagged constructs. The cell lysates were immunoprecipitated (*IP*) with anti-HA MoAb (12CA5), resolved by denaturing SDS-PAGE, and transferred to membranes. The blots were hybridized with anti-FLAG MoAb to detect co-precipitated huCOP1 proteins (*top panel*). Expression of HA-COP1 and of the deletion constructs in the different samples was determined by blotting of whole cell lysates with either anti-HA MoAb (*middle panel*) or with anti-FLAG MoAb (*bottom panel*). The *asterisk* indicates immunoglobulin heavy chains.

dopsis cells the presence of an intact COP1 protein is necessary to allow proteasome-dependent degradation of the HY5 transcription factor, in response to the appropriate extracellular signals (light to darkness switch). The molecular mechanisms through which atCOP1 acts is still unknown. It has been hypothesized that atCOP1 could function as a ubiquitin ligase of the RING family (29); however, ubiquitin ligase activity for atCOP1 has never been demonstrated. Ubiquitination of proteins requires the coordinated action of different components. An E1 enzyme forms a high energy thioesther bond with ubiquitin and transfers it to one of several E2 enzymes. E2 enzymes transfer ubiquitin to the substrate, in concert with an E3 enzyme that is responsible for substrate recognition (38). Several RING finger ubiquitin ligases have been shown to mediate E2-dependent self-ubiquitination *in vitro* (4), and we used this assay to test whether the RING domain of huCOP1 displays ubiquitin ligase activity. A glutathione *S*-transferase (GST) fusion protein with the RINGcc domains of COP1 was incubated in the presence of E1, ^{32}P-labeled ubiquitin, and UbcH5B as a source of E2 activity. The reaction products were analyzed by SDS-PAGE and autoradiography (Fig. 4*A*). Upon the addition of UbcH5B to the reaction, autoubiquitination of GST-RINGcc was detected (Fig. 4*A*, *right panel*). No ubiquitination was detectable when GST alone (Fig. 4*B*, *left panel*) was tested or when a RINGcc construct carrying a mutation in the zinc-binding residues of the RING finger was added (Fig. 4*B*, *GST-mutRINGcc*), indicating that the RING finger of COP1 is required for the E3 activity detected in this assay. Also full-length huCOP1 possessed ubiquitin ligase activity in this system, although slightly weaker than the RINGcc construct alone (not shown). As is the case for many RING finger proteins, huCOP1 was also ubiquitinated *in vivo* (see supplemental figure).

HuCOP1 Forms Complexes with Selected bZIP Transcription Factors—atCOP1 has been reported to directly interact with the bZIP *Arabidopsis* transcription factor HY5 (28). This interaction is critical for atCOP1-mediated regulation of HY5 transcriptional activity. We asked whether huCOP1 has the ability

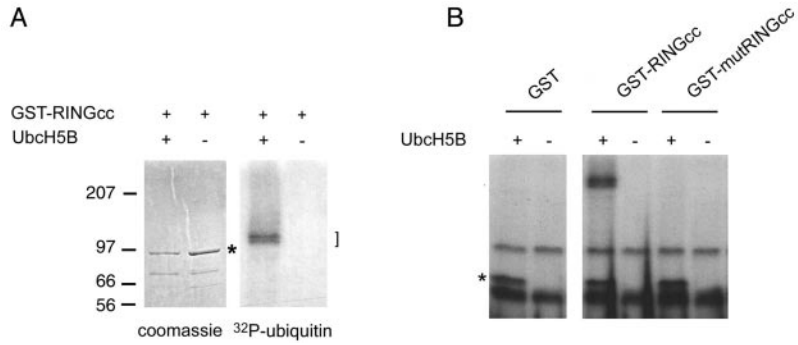

FIG. 4. **The RING domain of COP1 has ubiquitin-ligase activity.** *A*, the recombinant amino-terminal fragment of COP1 (RING and coiled-coil domains, GST-RINGcc) was tested *in vitro* for its ability to induce autoubiquitination in the presence of E1, E2, and ^{32}P-labeled ubiquitin. The reactions were analyzed by SDS-PAGE and autoradiography (*right panel*). Protein loading was controlled by Coomassie staining of the same gel (*left panel*; *asterisk* indicates GST-RINGcc recombinant protein). In the presence of the ubiquitin-conjugating enzyme UbcH5B (*first* and *third lanes*) discrete bands of mono- and di-ubiquitination (*square bracket*), as well as a high molecular weight smear of polyubiquitinated forms were detected in the sample containing both COP1 and the E2 UbcH5B. *B*, mutation of cysteine residues in the RING domain of COP1 abolished the ubiquitination activity of the recombinant protein (GST-mutRINGcc) *in vitro*. The experiments were performed as in *A*. Autoradiograms of the reactions resolved by SDS-PAGE are shown. Recombinant GST alone shows no activity (*left panel*), as well as the mutant COP1 construct (*right panel*, *third lane*), even in the presence of the E2 UbcH5B. The *asterisk* labels ubiquitin-charged UbcH5B.

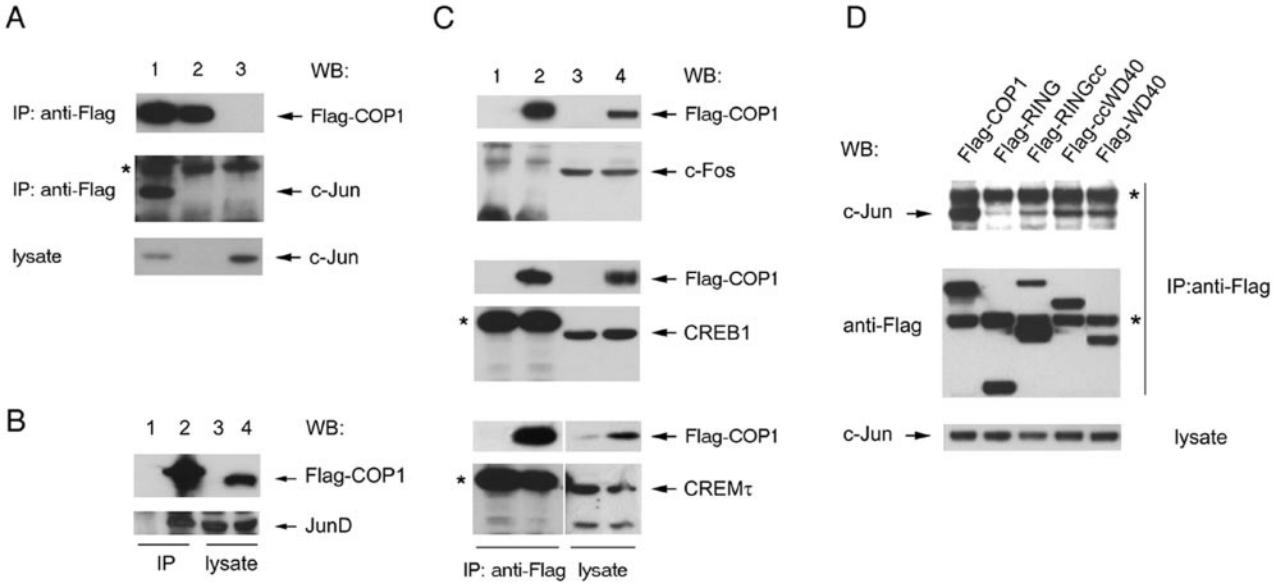

FIG. 5. **huCOP1 interacts with AP-1 transcription factors.** *A*, c-Jun co-precipitates with huCOP1. COS7 cells were transfected with FLAG-COP1 and c-Jun (*lane 1*), FLAG-COP1 alone (*lane 2*) or c-Jun alone (*lane 3*). Anti-FLAG immunoprecipitates (*IP*) and total cell lysates (*bottom panel*) were analyzed by Western blotting with anti-FLAG or anti-c-Jun antibodies as indicated. The *asterisk* indicates immunoglobulin heavy chains. *B*, JunD co-precipitates with huCOP1. The cells were transfected with JunD alone (*lanes 1* and *3*) or with JunD and FLAG-COP1 (*lanes 2* and *4*). Anti-FLAG immunoprecipitates (*lanes 1* and *2*) as well as total lysates (*lanes 3* and *4*) were analyzed by Western blotting with anti-FLAG or anti-JunD antibodies. *C*, huCOP1 does not co-precipitate with unrelated basic zipper transcription factors. The cells were transfected with the indicated transcription factors (c-Fos, Myc-tagged CREB1, or Myc-tagged CREMτ) in the absence (*lanes 1* and *3*) or the presence (*lanes 2* and *4*) of FLAG-COP1. Anti-FLAG immunoprecipitates (*lanes 1* and *2*) and total cell lysates (*lanes 3* and *4*) were analyzed by Western blotting with anti-FLAG, anti-c-Fos, or anti-Myc tag antibodies (to detect CREB1 and CREMτ). The *asterisks* indicate immunoglobulin heavy chains in immunoprecipitates. *D*, huCOP1 WD40 repeats are sufficient for the interaction with c-Jun. Full-length huCOP1 (FLAG-COP1) or the indicated deletion mutants were immunoprecipitated from COS7 cells in the presence of overexpressed c-Jun. Immunoprecipitates (*top* and *middle panels*) were analyzed by Western blotting with anti-c-Jun or anti-FLAG antibodies. c-Jun content in total cell lysates is shown in the *bottom panel*. The *asterisks* indicate immunoglobulin heavy chains in immunoprecipitates.

to interact with human bZIP transcription factors. We transfected FLAG-COP1 together with a panel of bZIP transcription factors and immunoprecipitated huCOP1 with anti-FLAG antibodies. huCOP1 co-precipitated selectively with Jun family members (c-Jun in Fig. 5*A*; JunD in Fig. 5*B*) but not with the other bZIP factors tested (c-Fos, CREB1, and CREMτ; Fig. 5*C*). JunD was also independently isolated as an interactor of huCOP1 (fused to the GAL4 DNA-binding domain) in a yeast two-hybrid screen of a human B cell library (not shown).

Co-immunoprecipitation experiments of c-Jun with the different deletion constructs of COP1 showed that the main site of interaction between COP1 and c-Jun lies in the WD40 domain, as reported for the binding of HY5 to atCOP1 (39), although an additional binding site is present in the coiled-coil region of the protein (Fig. 5*D*).

Although in *Arabidopsis* dimerization of atCOP1 has been reported to be necessary for the interaction with the HY5 transcription factor, in mammalian cells COP1 seems not to require dimerization to interact with c-Jun, as shown by the ability of the isolated WD40 domain to co-precipitate c-Jun (Fig. 4*D*) in the absence of homodimerization (Fig. 3).

A consensus motif whose integrity is essential for the interaction with atCOP1 has been identified in *Arabidopsis* proteins that bind to atCOP1, such as HY5 or STH (40). We found that

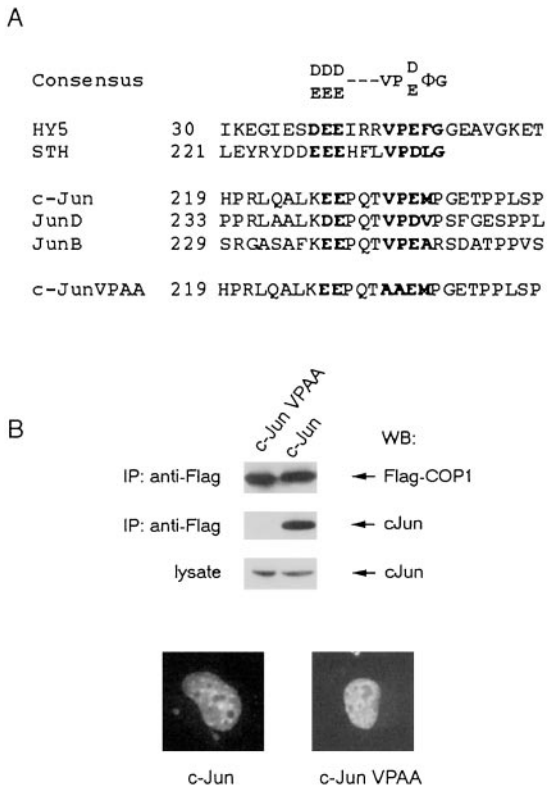

FIG. 6. **The interaction of huCOP1 with c-Jun is mediated by a conserved motif.** *A*, a conserved interaction motif has been identified in *A. thaliana* proteins (HY5 and STH) that interact with *Arabidopsis* COP1, indicated as *Consensus*. A similar motif is present in Jun family transcription factors. Φ is any hydrophobic amino acid. *B*, mutation of the consensus motif disrupts the interaction between huCOP1 and c-Jun. COS7 cells were transfected with full-length COP1 and either wild type c-Jun or c-JunVPAA, as indicated. Anti-FLAG immunoprecipitates (*IP*, *two top panels*) and total cell lysates were analyzed by Western blotting with anti-FLAG or anti-c-Jun antibodies. The c-JunVPAA protein carrying a mutation in the conserved interaction motif conserves the same nuclear localization of wild type c-Jun in COS7 cells, as demonstrated by indirect immunofluorescent analysis of wild type c-Jun (*bottom left panel*) and the VPAA c-Jun mutant (*bottom right panel*) expressed in COS7 cells.

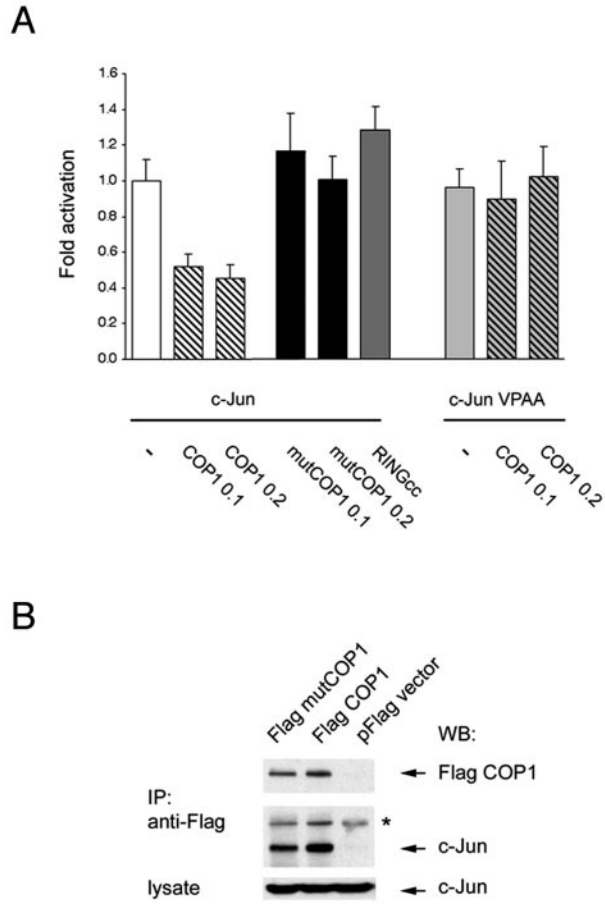

FIG. 7. **huCOP1 interacts functionally with c-Jun to regulate its transcriptional activity.** *A*, 293T cells were transfected with an AP-1 luciferase reporter construct, a β-galactosidase reporter construct, in the presence of either wild type c-Jun (*left bars*) or mutant c-Jun VPAA (*right bars*). In addition, the samples were transfected with empty vector (−) or the indicated microgram amounts of wild type huCOP1 (*hatched bars*) or huCOP1 with a RING mutation (*mutCOP1*, *black bars*). The total amount of DNA was kept constant with vector DNA. RINGcc (*dark gray bar*); the deletion mutant of huCOP1 missing the WD40 domain was expressed. Luciferase activity was normalized to β-galactosidase activity in each sample. The *bars* represent the means and standard deviations from three (RINGcc) or nine independent transfections, relative to the control sample (empty vector). *B*, huCOP1 RING mutant interacts with c-Jun. 293T cells were transfected with c-Jun and either wild type huCOP1 (FLAG COP1) or huCOP1 with a mutation in the RING domain (FLAG *mutCOP1*) or with empty vector. Anti-FLAG immunoprecipitates (*IP*) were blotted for c-Jun (*middle panel*). The *bottom panel* shows c-Jun content in total lysates. *Asterisk*, antibody heavy chain. *WB*, Western blot.

this motif is also highly conserved in Jun family proteins (Fig. 6*A*). To test the relevance of this motif for the interaction between c-Jun and huCOP1, we mutated to Ala two residues central to the motif (Val^{232}-Pro^{233}), resulting in the c-JunVPAA construct (Fig. 6*A*). This substitution was sufficient to completely abolish binding of c-Jun to COP1 (Fig. 6*B*), although it did not alter the subcellular distribution of the protein (Fig. 6*B*). In addition, c-JunVPAA remained transcriptionally active (Fig. 7*A*).

HuCOP1 Modulates AP-1-driven Transcription—To test the functional consequences of the observed interaction between c-Jun and huCOP1, we transfected 293T cells with a luciferase reporter gene under the control of an AP-1 promoter, together with c-Jun and increasing quantities of huCOP1. c-Jun-mediated transactivation was suppressed in a dose-dependent manner by the presence of wild type huCOP1 (Fig. 7*A*, *hatched bars versus white bar*). The inhibitory effect of huCOP1 depended on the interaction with c-Jun, as demonstrated by the inability of huCOP1 to interfere with the transcriptional activity of c-JunVPAA (Fig. 7*A*, *bars* on the *right*) and by the absence of a suppressive effect when the RINGcc deletion mutant of COP1, lacking the main c-Jun binding site, was transfected (Fig. 7*A*). Interestingly, the inhibitory effect required the presence of an intact COP1 RING domain, although the RING domain is not involved in binding of COP1 to c-Jun. The RING mutant of COP1, which carries two Cys to Ala substitutions in the RING domain, did not affect c-Jun transactivation activity (Fig. 7*A*, *black bars*), although it still bound c-Jun in a co-immunoprecipitation assay (Fig. 7*B*), indicating that the inhibitory action of huCOP1 on c-Jun transcriptional activity is not due to a simple steric hindrance effect. To test whether huCOP1 could also affect c-Jun-dependent transactivation of a natural AP-1 promoter, we transfected 293T cells with a luciferase reporter gene comprising the basal proximal promoter and the AP-1 enhancer region of the urokinase (u-PA) gene (34) (Fig. 8*A*, *right bars*). Transfection of huCOP1 (*hatched bars*) reduced c-Jun-dependent transactivation of the AP-1 enhancer but had no effect on the activity of the basal proximal u-PA promoter, which is regulated by Sp family proteins (Fig. 8*A*, *left bars*) (34). The ability of huCOP1 to modulate c-Jun activity in a physiological promoter context was further tested by the effect of huCOP1 expression on the mRNA levels of known AP-1 target genes, such as u-PA itself and MMP1 (41–43). These

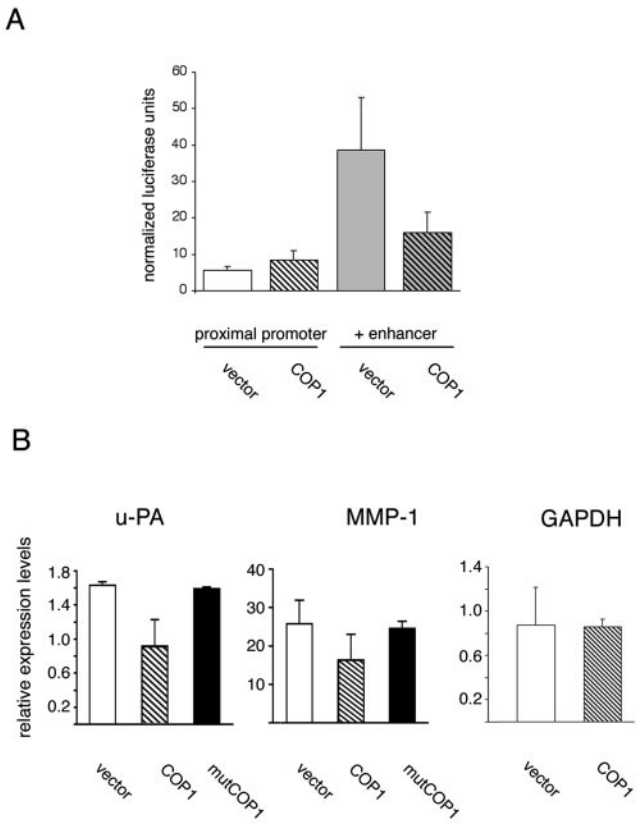

FIG. 8. **huCOP1 regulates the expression of AP-1 target genes.** *A*, huCOP1 regulates transcription from a natural AP-1-driven promoter. The cells were transfected with either COP1 (0.2 μg, *hatched bars*) or empty vector, in the presence of a luciferase reporter construct comprising the proximal basal promoter of the urokinase gene (*left bars*) or the proximal promoter and the AP-1-dependent urokinase enhancer (*right bars*). The luciferase activity was normalized to β-galactosidase activity in each sample. The *bars* represent the averages and standard deviations from six independent transfections. *B*, huCOP1 down-regulates mRNA levels of AP-1 target genes. HeLa cells (*left* and *right panels*) or 293T cells were transfected with c-Jun (*left* and *right panels*) or c-Jun plus c-Fos (*middle panel*), in the presence of either wild type COP1 (*hatched bars*) or RING mutant COP1 (*black bars*). mRNA levels for urokinase (u-PA), MMP1, and GAPDH were estimated in total RNA samples by fluorogenic real time reverse transcriptase-PCR. Shown are expression levels, normalized to 18 S amplification levels in each sample and calculated relative to the expression level of the target gene in the mock transfected sample. The bars represent the means and standard deviations from six (u-PA and GAPDH) or three (MMP-1) independent transfections.

genes were chosen among the characterized c-Jun targets for their c-Jun-dependent inducibility in the cellular systems used in this study (293T or HeLa cells). The cells were transfected in triplicate with c-Jun or c-Jun plus c-Fos to induce AP-1-dependent expression of u-PA or MMP1, respectively. The samples were also transfected with either 0.2 μg of huCOP1, 0.2 μg of the RING mutant of COP1, or empty vector. After 48 h, total RNA was extracted from each sample, and the relative mRNA levels for u-PA and MMP1 were estimated by quantitative real time reverse transcriptase-PCR. Overexpression of huCOP1 resulted in decreased inducibility of the analyzed AP-1 target genes (Fig. 8*B*, *hatched bars*) but did not alter the levels of the control gene (Fig. 8*B*, *GAPDH*). Conversely, expression of mutant COP1 was ineffective in regulating AP-1-dependent expression of the target genes (Fig. 8*B*, *black bars*).

HuCOP1 Does Not Directly Affect c-Jun Ubiquitination—Because the integrity of the RING domain is necessary not only for huCOP1 inhibitory activity on AP-1-dependent transcription but also for huCOP1 ubiquitin ligase activity, we tested the hypothesis that c-Jun function may be down-regulated following ubiquitination of c-Jun by huCOP1. In an *in vitro* assay, the addition of purified E1, UbcH5B, and huCOP1 alone was not sufficient to induce ubiquitination of recombinant, bacterially expressed c-Jun (not shown). In addition, overexpression of huCOP1 in 293T cells did not consistently affect c-Jun steady state levels in cell lysates, nor its half-life, as measured by pulse-chase experiments (not shown), indicating that the transcriptional inhibition activity of huCOP1 may be independent of its ubiquitin ligase properties.

DISCUSSION

In this paper we report the cloning and characterization of the human orthologue of the *Arabidopsis* RING protein at-COP1, which plays a fundamental role in the control of gene expression during plant development (26) by relaying light/dark-derived signals to the transcriptional machinery. Human COP1 shows a high degree of sequence conservation and domain organization with its plant counterpart. We found that transfected huCOP1 distributed to the cytoplasm and the nucleus of cells and that nuclear localization was directed by a NLS located in the amino-terminal region of the protein. This dual distribution may be important for huCOP1 function, because it has been demonstrated to be essential, in *Arabidopsis*, for regulating COP1 activity in response to extracellular stimuli. Consistently, a partial, chimeric mouse/human COP1 construct responded to light/dark stimuli by changing its subcellular localization pattern when introduced in *Arabidopsis* cells, pointing to a close functional conservation between the mammalian and the plant protein (44).

The putative coiled-coil region (amino acids 203–305) and the seven WD40 motifs (amino acids 405–731) of huCOP1 represent potential interaction domains for protein partners. The discrete nuclear pattern of huCOP1 depended on the presence of these domains and is compatible with distribution to specific subnuclear compartments, as has been described for other RING proteins, such as PML (45). The coiled-coil region was also involved in huCOP1 homodimerization, a property shared by atCOP1 (39). The ability of huCOP1 to establish several protein-protein interactions is further suggested by our preliminary gel filtration studies (not shown) indicating that COP1 may be present *in vivo* in a high molecular weight complex. These data are consistent with the notion that the assembly in large macromolecular structures is a frequent characteristic of proteins containing a RING domain (1).

The striking degree of structural conservation of huCOP1 with the *Arabidopsis* counterpart suggested the interesting possibility of a functional conservation as well. Central to *Arabidopsis* COP1 biological function in seedling development is the regulation of a bZIP protein, the photomorphogenic transcription factor HY5. The inhibitory activity of atCOP1 on HY5-dependent transcription requires the direct interaction of the two proteins through a specific binding motif (acidic residues followed by VPD/E and a hydrophobic amino acid), present in several COP1-binding proteins (40). We identified a similar amino acid motif in the mammalian bZIP proteins c-Jun and JunD, members of the AP-1 family of transcription factors. AP-1 factors convert multiple extracellular stimuli, such as growth factors, cytokines, and cellular stress, into a variety of transcriptional responses. AP-1 target genes have been implicated in many diverse biological processes, including oncogenesis, cell survival, proliferation, and differentiation (46, 47), making the AP-1 family one of the most complex systems of transcriptional control. Protein-protein interactions play an important role in regulating AP-1-dependent transcription and in controlling the specificity of AP-1 transcriptional responses (41, 48).

The COP1-binding motif we identified in c-Jun and JunD mediated the interaction between these factors and huCOP1 and suggested that this highly conserved pathway is involved in the regulation of Jun-dependent transcription. Consistently, expression of huCOP1 inhibited in a dose-dependent fashion c-Jun-dependent transcription both of synthetic promoter-reporter constructs and of known AP-1 target genes. Further, this effect required the integrity of the COP1-binding consensus.

The molecular basis of *Arabidopsis* COP1 suppressive activity on transcription are unknown; however, it has been hypothesized that COP1 may function as a ubiquitin ligase that directly targets transcription factors for degradation, based on the presence of the RING finger domain and on the ability of atCOP1 to permit degradation of HY5 in response to the adequate extracellular stimulus (29). We found that the integrity of the RING domain is important for huCOP1 inhibitory activity on AP-1-dependent transcription, and this prompted us to test a possible role of huCOP1 in inducing the degradation of c-Jun.

Overexpression of huCOP1 in two different cellular systems (COS7 and 293T cells) did not result in a decrease of c-Jun steady state levels in total cell lysates, nor in a reduction of c-Jun half-life, as analyzed by pulse-chase experiments (not shown). Consistently, in our *in vitro* ubiquitination assay we could demonstrate that the RING of huCOP1 has indeed ubiquitinating activity toward COP1 itself, but it is not capable of inducing ubiquitination of a recombinant c-Jun protein, in the presence of purified E1 and UbcH5B (not shown). Our findings raise the possibility that ubiquitination of c-Jun by huCOP1 may occur only in the presence of another yet to be identified component of the ubiquitin ligase complex requiring the integrity of huCOP1 RING finger domain for its function. This hypothesis is supported by the recent findings by Hardtke *et al.* (49) that the *Arabidopsis* COP1-interacting protein CIP8 promotes ubiquitination of HY5 *in vitro*, suggesting that ubiquitination of HY5 *in vivo* could require the formation of a multimolecular complex that includes both COP1 and CIP8.

Alternatively, we cannot rule out the possibility that signal-dependent post-translational modifications of c-Jun may be necessary to render the molecule susceptible to ubiquitination or to proteasome-dependent proteolysis. Furthermore, a specific (and still unknown) extracellular cue could be required to trigger COP1-dependent degradation of c-Jun in mammalian cells, in analogy to the dark/light signals that regulate atCOP1 function in *A. thaliana*.

On the other hand, our observation that huCOP1 interferes with c-Jun transcriptional activity in the presence of constant levels of total c-Jun protein indicates that inhibition of AP-1-dependent transcription occurs independently of protein degradation, as reported for other RING finger proteins implicated in transcriptional regulation (see Refs. 1 and 7 for reviews). The requirement for an intact RING domain, in this case, may reflect the importance of protein-protein interactions established by the RING in mediating transcriptional repression rather than its role in protein ubiquitination.

The identification of huCOP1-interacting proteins will be of great importance to fully understand the function of huCOP1 in regulating c-Jun transcriptional activity. A genetic and functional interaction between COP1 and the CSN has been described in *Arabidopsis*, where loss of function mutants of the CSN show morphogenic alterations virtually indistinguishable from those of COP1 loss of function mutants (50). The CSN has been implicated in the correct subcellular localization of COP1, because atCOP1 is excluded from the nucleus in CSN mutants (51). It will be of interest to investigate the relationship between huCOP1 and the CSN. In mammalian cells, the CSN has been implicated by different laboratories (52–54) as well as by ours (31) in the modulation of AP-1-dependent transcription. The molecular mechanisms through which the CSN affects c-Jun function are still debated and may include post-translational modifications of c-Jun (55). Whether a functional interaction with huCOP1 plays a role in this aspect of CSN activity is an interesting possibility that still remains to be tested.

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Characterization of Human Constitutive Photomorphogenesis Protein 1, a RING Finger Ubiquitin Ligase That Interacts with Jun Transcription Factors and Modulates Their Transcriptional Activity

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