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Functional expression of the Wilson disease protein reveals mislocalization and impaired copper-dependent trafficking of the common H1069Q mutation

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ABSTRACT **Wilson disease is an autosomal recessive disorder of hepatic copper metabolism caused by mutations in a gene encoding a copper-transporting P-type ATPase. To elucidate the function of the Wilson protein, wild-type and mutant Wilson cDNAs were expressed in a Menkes copper transporter-deficient *mottled* fibroblast cell line defective in copper export. Expression of the wild-type cDNA demonstrated trans-Golgi network localization and copper-dependent trafficking of the Wilson protein identical to previous observations for the endogenously expressed protein in hepatocytes. Furthermore, expression of the Wilson cDNA rescued the *mottled* phenotype as evidenced by a reduction in copper accumulation and restoration of cell viability. In contrast, expression of an H1069Q mutant Wilson cDNA did not rescue the *mottled* phenotype, and immunofluorescence studies showed that this mutant Wilson protein was localized in the endoplasmic reticulum. Consistent with these findings, pulse–chase analysis demonstrated a 5-fold decrease in the half-life of the H1069Q mutant as compared with the wild-type protein. Maintenance of these transfected cell lines at 28°C resulted in localization of the H1069Q protein in the trans-Golgi network, suggesting that a temperature-sensitive defect in protein folding followed by degradation constitutes the molecular basis of Wilson disease in patients harboring the H1069Q mutation. Taken together, these studies describe a tractable expression system for elucidating the function and localization of the copper-transporting ATPases in mammalian cells and provide compelling evidence that the Wilson protein can functionally substitute for the Menkes protein, supporting the concept that these proteins use common biochemical mechanisms to effect cellular copper homeostasis.**

Wilson disease (WD) is an inherited disorder of copper metabolism characterized by hepatic cirrhosis and neuronal degeneration caused by a marked impairment in biliary copper excretion (1, 2). Consistent with this clinical phenotype, the WD gene has been cloned and shown to encode a copper-transporting P-type ATPase with primary expression in the liver (3–5). Immunocytochemical studies have demonstrated that the Wilson protein is localized in the trans-Golgi network (6, 7) and exhibits a copper-dependent translocation to a cytoplasmic vesicular compartment (6). Recent studies have confirmed that the amino terminus of this protein binds copper (8, 9), and expression of the human Wilson protein in *ccc2*Δ yeast lacking the homologous ATPase (10) and in the LEC rat, an animal model of WD (11, 12), demonstrates restoration of cuproprotein synthesis, indicating a direct role for the Wilson protein in copper transport (6, 13). Together these data suggest a model in which the Wilson protein resides in the trans-Golgi network and recycles to a vesicular compartment under high cellular copper conditions, where it functions to transport copper into the secretory compartment for incorporation into nascent cuproenzymes and to export copper from the cell. Similar observations have been made for the homologues copper transporting ATPase involved in the X-linked disorder Menkes disease (14). These findings suggest common mechanisms of cellular trafficking and function for these two copper transporters.

Despite elucidation of the cell biology of the Wilson protein, little data is currently available regarding the structural determinants of protein function. Disease mutations can serve as a guide for such analyses, and clinical studies have revealed that an H1069Q mutation represents up to 37% of WD alleles in patients of Eastern European descent (15, 16). Previous studies in *Saccharomyces cerevisiae* have indicated that the Wilson H1069Q mutant protein and the equivalent mutant of the homologous Menkes protein are defective in copper transport (6, 17); however, characterization of the cell biology defect in yeast has been limited because post-Golgi sorting compartments are not morphologically conserved between yeast and mammalian cells. Consequently, the current study was undertaken to elucidate the function of the Wilson protein in mammalian cells and to define the molecular basis of the defect in patients with the H1069Q mutation.

MATERIALS AND METHODS

Cell Culture and Antibodies. The murine *mottled* fibroblast cell line 802–1 (Mo −/Y, Mt −/+), and the corresponding wild-type cell line 802–5 (Mo +/Y, Mt −/+) were a gift from Richard Palmiter (University of Washington School of Medicine, Seattle). Cell lines were maintained in DMEM containing 10% fetal bovine serum and supplemented with penicillin/streptomycin. The polyclonal antiserum to the Wilson protein was described previously (6, 18). Murine mAbs to protein disulfide isomerase and ADP ribosylation factor (ARF) were purchased from StressGen Biotechnologies (Victoria, Canada) and Affinity BioReagents (Neshanic Station, NJ), respectively. Secondary antibodies conjugated with fluorescein isothiocyanate or lissamine rhodamine isothiocyanate were from Jackson ImmunoResearch.

Subcloning and Mutagenesis of the Wilson cDNA. The ORF of the human WD protein was isolated from pYES2/hWD (6) by restriction digest with *Bam*HI and subcloned into the *Bam*HI site of the cytomegalovirus promoter-based mammalian expression vector pcDNA3.1 (Invitrogen). Site-directed

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Abbreviations: WD, Wilson disease; ARF, ADP ribosylation factor.
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mutagenesis was performed by the PCR with Klentaq polymerase (CLONTECH) and oligonucleotide primer pairs corresponding to the H1069Q and H1069A mutations along with flanking 5′ sense and 3′ antisense oligonucleotides (19). Ligated vectors were used to transform Top10F′ (Invitrogen) and screened by restriction digest. The presence of specific mutations and the fidelity of the cDNA sequence were verified by automated fluorescent sequencing (Perkin–Elmer) according to the manufacturer's recommendations.

Cell Transfection. For stable transfections, murine *mottled* fibroblasts (802–1) were electroporated by using a GenePulser apparatus (Bio-Rad) according to the manufacturer's recommendations. Cells (10^7) in 300 μl of DMEM were mixed with 20 μg of linearized Wilson plasmid DNA and 4 μg of linearized pZeoSV2 (Invitrogen) in 100 μl of Hepes-buffered saline, pH 7.05 in a 0.4-cm gap cuvette on ice. Cells were pulsed at 0.25 kV/1,000 μF, allowed to recover for 48 hr in complete growth media, and placed into selective media [complete growth media supplemented with 0.5 mg/ml zeocin (Invitrogen)]. Single colonies were isolated 10–14 days after selection with cloning cylinders and expanded for screening and further analysis.

Immunoblotting, Immunoprecipitation, and Immunofluorescence. Immunoblotting was performed as previously described, by using the protease inhibitors aprotinin, phenylmethylsulfonylfluoride, leupeptin, and pepstatin (6). Before immunoprecipitation, cells were pulsed with 300 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine and chased with media containing 50 mM methionine and cysteine. Cells were lysed in PBS, pH 7.4 containing 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and protease inhibitors for 20 min at 4°C followed by centrifugation for 20 min at 16,000 $\times$ *g* at 4°C. The protein concentration of the precleared supernatant was determined by the method of Bradford (20), and equivalent amounts of protein from each time point were immunoprecipitated with Wilson antibody as described (21). Immunofluorescence was performed as described (6) with pretreatment of cells with either 50 μM bathocuproine disulfonate (Sigma) for 16–24 hr or 400 μM $CuCl_2$ for 2–3 hr for the −Cu and +Cu conditions, respectively.

Copper Retention and Toxicity Assays. To evaluate ^{64}Cu retention, 2×10^5 cells were plated in triplicate in 35-mm tissue culture dishes in complete growth media and incubated with 6,000,000 cpm ^{64}Cu (specific activity 30–80 mCi/mg) for 72 hr. Cells were washed extensively in PBS and lysed in 1% Nonidet P-40, 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 10% glycerol supplemented with protease inhibitors for 20 min at 4°C. Protein determination and evaluation of gamma radiation was measured in duplicate on an aliquot of the total cell lysate, and copper retention was calculated as cpm per μg of protein. Copper toxicity was evaluated by using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Sigma) as previously described (22, 23) except that cell viability was assessed after 3 days of growth.

RESULTS

The wild-type and mutant H1069Q Wilson cDNAs were cloned into a mammalian expression vector to examine copper-dependent trafficking by the wild-type WD protein and the Wilson protein harboring the common H1069Q patient mutation. To distinguish effects of the loss of the histidine residue from the gain of a glutamine residue, an H1069A Wilson cDNA mammalian expression plasmid also was generated. To evaluate the functional significance of Wilson cDNA expression, a stable mammalian expression system was developed in the murine *mottled* fibroblast cell line 802–1 (23), which is defective in the mouse homologue of the Menkes disease gene. The *mottled* cell line was derived from mutant mice carrying the ATP7a$^{Mo\text{-}br}$ allele of the murine Menkes gene, which has been shown to contain a 6-bp deletion predicted to delete two amino acids in a conserved region of the protein resulting in the severe phenotype similar to classical Menkes disease (24–26). In addition, the *mottled* cell line used here was heterozygous for deletion of the murine metallothionein I and II genes, which previously was shown to increase the sensitivity of these cells to copper, thus permitting a rapid functional assay for the Menkes gene (23).

The wild-type Wilson cDNA was stably transfected into *mottled* fibroblasts, and individual clones were selected and expanded for further analysis. Immunoblot analysis with Wilson antisera of lysates from two independent clones demonstrated expression of a 165-kDa protein identical in size to the Wilson protein endogenously expressed in HepG2 cells (Fig. 1*A*). Indirect immunofluorescence on the parental cell line 802–1 with Wilson antibody showed nonspecific staining (Fig. 1*B*, *Left*), and indirect immunofluorescence on *mottled* fibroblast clone 4 expressing the Wilson protein demonstrated trans-Golgi network localization in low copper media, which redistributed to a vesicular compartment in high copper media. The trans-Golgi marker protein ARF did not change localization in response to a change in the media copper concentration (Fig. 1*B*, *Right*).

To investigate the copper export function of the expressed Wilson protein, ^{64}Cu retention by the stably transfected clones and the parental cell lines was examined. The parental 802–1 *mottled* fibroblast line accumulated approximately 3-fold higher amounts of ^{64}Cu than its corresponding wild-type fibroblast line 802–5 during the assay period (Fig. 1*C*). Expression of the wild-type Wilson protein in two independent *mottled* clones reduced the accumulation of ^{64}Cu to levels approximately 3-fold less than that of the wild-type line 802–5, consistent with the role of this protein in copper export. Because the accumulation of copper within the cell is associated with reduced cell viability (23), this characteristic was examined in an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide-based assay by using the parental and transfected cell lines. The 802–1 *mottled* fibroblast exhibited a 50% decrease in cell viability with 10 μM $CuCl_2$ in the cellular media, whereas the corresponding wild-type fibroblast line 802–5 remained at approximately 100% viability (Fig. 1*D*). Consistent with the observations on copper retention shown in Fig. 1*C*, the *mottled* fibroblast clone 4 expressing the Wilson protein also demonstrated 100% viability under the same conditions. As the copper toxicity profile accurately reflected the ^{64}Cu retention of the cell lines, copper export was evaluated by using this copper toxicity profile for the remainder of the study.

These studies demonstrated a functional expression system for the Wilson protein and thus *mottled* fibroblast clones expressing the Wilson H1069Q and H1069A mutant proteins were generated and examined for protein function. To discount integration effects on the observed phenotype, at least two clones were tested in each experiment with similar results. Immunoblot analysis of lysates from H1069Q and H1069A clones with Wilson antiserum demonstrated expression of a 165-kDa protein, with consistently lower steady-state levels of both mutant proteins as compared with the wild-type protein (Fig. 2*A*, lanes 3 and 4 versus lane 2). The copper toxicity profile of the transfected cell lines demonstrated 100% cell viability of the *mottled* clone expressing the wild-type Wilson protein (Fig. 2*B*), whereas cell viability of *mottled* clones expressing the H1069Q and H1069A decreased to approximately 50% at 10 μM $CuCl_2$ in the cellular media, similar to what was observed for the parental *mottled* fibroblast. These observations were not caused by variable expression within a given cell line as immunofluorescence revealed that all cells in a culture expressed the transfected protein (data not shown).

As these functional studies demonstrated that neither the H1069Q nor the H1069A mutant proteins rescue the *mottled*

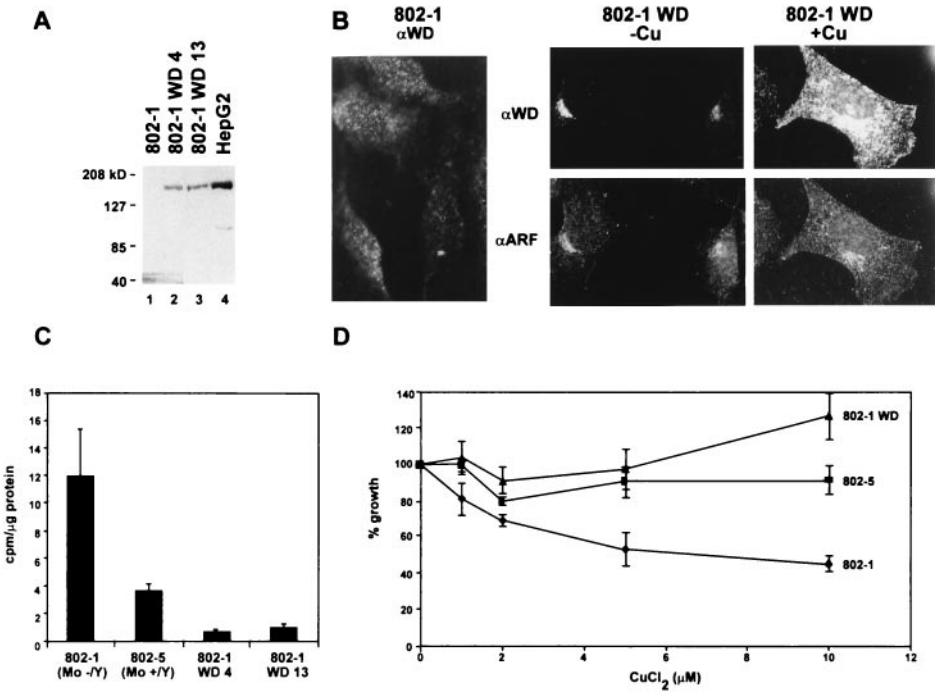

FIG. 1. (*A*) Immunoblot of lysates from *mottled* fibroblasts (802–1) stably transfected with the wild-type Wilson cDNA. Protein (125 μg) from the parental cell line (lane 1) or two independent clones (lanes 2 and 3) with HepG2 lysate as a positive control (lane 4) were separated by SDS/7.5% PAGE, transferred to nitrocellulose, incubated with Wilson antiserum, and developed with chemiluminescence. (*B*) Indirect immunofluorescence localization of the wild-type Wilson protein (WD) and ARF with or without copper treatment in the stably transfected *mottled* fibroblast clone 4, with the parental 802–1 cell line as a negative control. (×600) (*C*) ^{64}Cu retention by *mottled* fibroblasts (802–1 Mo −/Y), wild-type fibroblasts (802–5 Mo +/Y), and two independent clones of *mottled* fibroblasts stably transfected with the Wilson cDNA (802–1 WD 4 and 802–1 WD 13). Cells were incubated with ^{64}Cu for 72 hr and processed as described in *Materials and Methods*. (*D*) Copper toxicity profile of *mottled* fibroblasts (802–1), wild-type fibroblasts (802–5), and *mottled* fibroblast clone 4 stably transfected with the Wilson cDNA (802–1 WD). Cells were grown in media supplemented with increasing concentrations of copper and assessed for viability at 72 hr by using an 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide-based assay as described in *Materials and Methods*.

phenotype, the localization of these mutant proteins was investigated by using indirect immunofluorescence. Both Wilson protein mutants H1069Q and H1069A were localized in the endoplasmic reticulum as evidenced by colocalization with the marker protein disulfide isomerase (Fig. 3*A*). A small proportion of cells expressing H1069A demonstrated trans-Golgi network staining (data not shown). As endoplasmic reticulum localization may represent a pathway for turnover of misfolded proteins, pulse–chase biosynthetic studies of the Wilson protein were performed (Fig. 3*B*). Both the Wilson protein endogenously expressed in HepG2 cells and the wild-type Wilson protein stably expressed in the *mottled* fibroblast exhibited a half-life of approximately 8 hr. In contrast, the Wilson H1069Q and H1069A proteins demonstrated half-lives of approximately 1.5 and 2 hr, respectively. The difference in expression level between the wild-type and mutant proteins was entirely caused by turnover as metabolic labeling revealed similar rates of synthesis for each protein (data not shown).

These metabolic studies suggested a defect in protein folding that may be bypassed at lower temperatures. To evaluate the temperature sensitivity of the Wilson mutant proteins, the stably transfected *mottled* clones were maintained at 28°C and examined for protein function. Immunoblot of lysates from cells maintained at 28°C consistently demonstrated an increase in the steady-state level of the H1069Q and H1069A proteins when directly compared with the wild-type Wilson protein at each temperature (Fig. 4*A*; compare with Fig. 2*A*). Indirect immunofluorescence on a *mottled* clone expressing the wild-type Wilson protein at 28°C demonstrated perinuclear staining in the low copper state and movement into a vesicular compartment in the high copper state (Fig. 4*B*), indicating that the cellular copper trafficking factors are functional at the lowered temperature. Immunofluorescence on clones expressing the Wilson H1069Q protein showed rescue of trans-Golgi network localization, with approximately 20% of cells demonstrating colocalization with ARF (Fig. 4*C*, *Left*). However, when exposed to high media copper concentrations, the trans-Golgi network localization of the H1069Q protein did not change (Fig. 4*C*, *Right*). Indirect immunofluorescence experiments on clones expressing the Wilson H1069A protein demonstrated complete rescue of the trans-Golgi network localization in a low copper media (Fig. 4*D*, *Left*), but again no change in the localization when the cells were exposed to a high copper media (Fig. 4*D*, *Right*).

DISCUSSION

The data in this paper demonstrate the development of a stable expression system in a mammalian cell line that can be used to evaluate both the function and localization of the human copper-transporting ATPases. Expressed in this system, the wild-type Wilson protein rescued the *mottled* phenotype, consistent with the function of the Wilson protein in copper export. Furthermore, the Wilson protein was localized in the trans-Golgi network and exhibited copper-dependent recycling to a cytoplasmic vesicular compartment (Fig. 1), consistent with previous studies on the cell biology of the Wilson protein in HepG2 cells (6) and similar to observations for the homologous Menkes protein in human and rodent cell lines (14, 18, 27). Plasma membrane localization of the Wilson protein was not observed, supporting previous findings for the endogenously expressed protein in HepG2 cells (6). Interestingly, in transient transfections in COS7 cells, the Wilson protein was localized to the trans-Golgi network, cytoplasmic vesicles, and the plasma membrane in a high copper state (data not shown), suggesting that saturable components of the

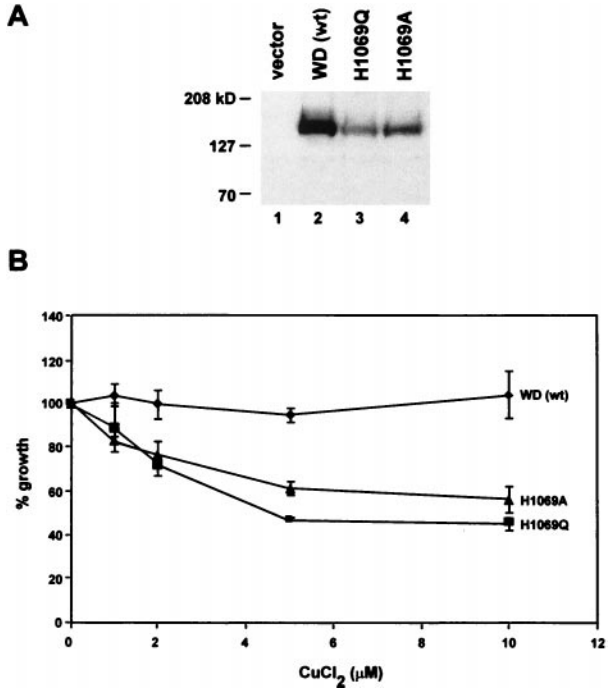

FIG. 2. (*A*) Immunoblot of lysates from *mottled* fibroblast clones stably transfected with Wilson cDNA mutants H1069Q and H1069A. Protein (125 μg) from cells transfected with vector alone (lane 1), wild-type Wilson cDNA (lane 2), or Wilson cDNA mutants (lanes 3 and 4) were separated by SDS/7.5% PAGE, transferred to nitrocellulose, and analyzed by chemiluminescence. (*B*) Copper toxicity profile of *mottled* fibroblast clones stably transfected with the wild-type Wilson cDNA or mutants H1069Q and H1069A.

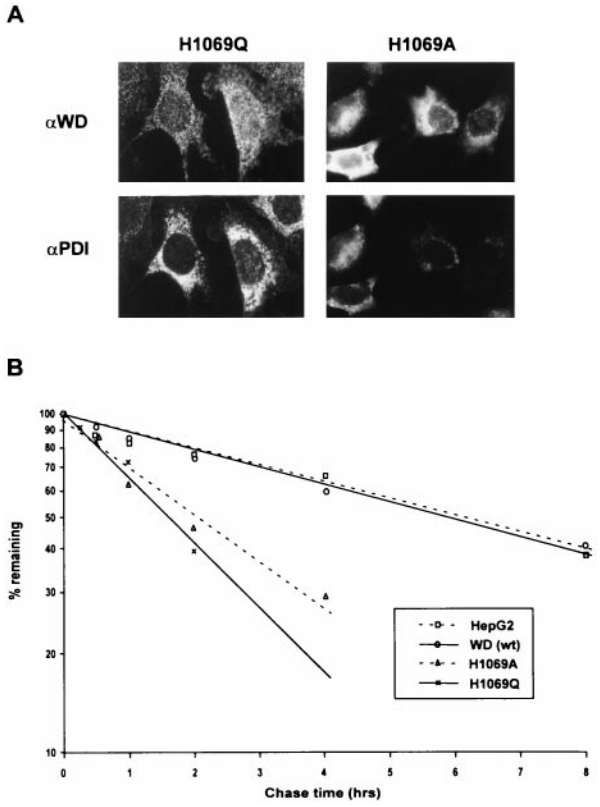

FIG. 3. (*A*) Immunofluorescence on *mottled* fibroblast clones stably transfected with Wilson cDNA mutants H1069Q and H1069A without copper treatment. Cells were processed for indirect immunofluorescence by using antibodies against the WD protein or protein disulfide isomerase. (×600.) (*B*) Pulse–chase labeling of Wilson protein endogenously expressed in HepG2 cells or stably expressed in *mottled* fibroblast clones as the wild-type protein or mutants H1069Q and H1069A. Cultures were pulsed for 30 min with [^{35}S]methionine and [^{35}S]cysteine and then chased for the indicated times in media containing excess methionine and cysteine. Immunoprecipitates of cell lysates were analyzed by fluorography after SDS/7.5% PAGE and were quantified by using a Phosphorimager (Molecular Dynamics).

cellular trafficking machinery (28) may be involved in the copper-dependent recycling of the protein. The nature of the post-Golgi compartment involved in copper transport is unknown but has been shown to be distinct from compartments involved in processes such as lysosomal enzyme sorting or receptor-mediated endocytosis (6, 14). The mechanism of copper-mediated trafficking of such vesicles is also unclear but may involve both energy transduction coordinated with copper movement across the membrane as well as interactions of the cellular sorting machinery with motifs in the carboxyl-terminal cytoplasmic domain, which are highly conserved in both the Menkes and Wilson proteins (6, 14).

The characterization of the H1069Q Wilson protein in the *mottled* fibroblast expression system suggests a mechanism for disease in patients harboring this mutant allele. The localization of the H1069Q Wilson protein to the endoplasmic reticulum (Fig. 3*A*) and the corresponding 5-fold decrease in the half-life of the mutant protein (Fig. 3*B*) explain the lower steady-state level of protein detected in the stable transfectants by immunoblot analysis (Fig. 2*A*) and the lack of detectable function of the expressed protein (Fig. 2*B*). These findings are analogous to previous observations for disease-causing mutations in another polytopic ion transporter, the cystic fibrosis transmembrane conductance regulator (CFTR) (29, 30). The restoration of trans-Golgi network localization of the mutant Wilson proteins at 28°C (Fig. 4 *C* and *D*, *Left*) is comparable to CFTR ΔF508, which properly refolds and bypasses the endoplasmic reticulum quality control machinery at lowered temperatures (31). This rescue of localization directly parallels the increased steady-state level of mutant proteins in the immunoblot analysis of lysates from cells maintained at 28°C (Fig. 4*A*), suggesting that constraints on folding are reduced at the lowered temperature, and consequently the mutant proteins are not degraded via the endoplasmic reticulum. Because many of the identified Wilson patient mutations have been point mutations in residues for which no specific function has been assigned (14, 15), the current findings on the misfolding and degradation of the H1069Q mutant protein may describe a more general model for WD analogous to cystic fibrosis (29) in which various amino acid substitutions result in an abnormally folded protein that is rapidly degraded and hence nonfunctional. In support of this concept, recent studies on an exon splice mutation of the Menkes gene reveal that this mutant ATPase also is localized to the endoplasmic reticulum (32).

In addition to the effect on protein processing, mutation of histidine 1069 in the Wilson protein abolishes copper-dependent recycling in high media copper conditions (Fig. 4 *C* and *D*, *Right*) even though the mutant proteins correctly localize to the trans-Golgi network in low copper conditions at 28°C. These findings indicate that histidine 1069 plays a critical role in the copper-dependent trafficking of the Wilson protein and suggest that pharmacologic interventions to overexpress and hence deliver some H1069Q mutant protein to the correct cellular location, as has been proposed for the treatment of cystic fibrosis transmembrane conductance regulator ΔF508 (33, 34) may not be clinically effective because the mutant protein does not exhibit the same trafficking activity as the wild-type protein. Interestingly, histidine 1069 is part of a SEHPL motif that is conserved among all copper-transporting ATPases (35) and is analogous to regions in the calcium-transporting P-type ATPases that interact with proteins to

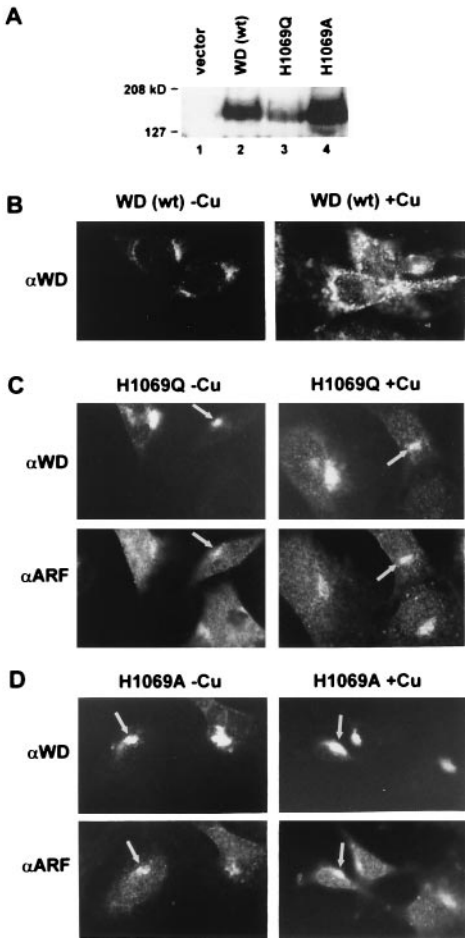

FIG. 4. (A) Immunoblot analysis of lysates from *mottled* fibroblast clones maintained at 28°C. Protein (125 μg) from cells stably transfected with vector alone (lane 1), wild-type Wilson cDNA (lane 2), or Wilson cDNA mutants (lanes 3 and 4) were separated by SDS/7.5% PAGE, transferred to nitrocellulose, and analyzed by chemiluminescence. (*B*) Immunofluorescence on wild-type Wilson clone 4 with and without copper treatment by using the Wilson protein antibody. (×600.) (*C*) Immunofluorescence on a Wilson H1069Q clone with and without copper treatment by using antibodies against the Wilson protein or ARF. Arrows indicate trans-Golgi network localization in corresponding cells. (×600.) (*D*) Immunofluorescence on a Wilson H1069A clone with and without copper treatment by using antibodies against the Wilson protein or ARF. Arrows indicate trans-Golgi network localization in corresponding cells. (×600.)

modulate the affinity of ion binding (36, 37). The relevance of copper-dependent trafficking to copper transport could not be explored because at 28°C no significant difference in viability, copper accumulation, or copper export was observed between the parent cell line and *mottled* fibroblasts (data not shown). Future studies to address the relationship between copper-dependent trafficking and copper export thus will be highly relevant to our understanding of potential disease treatments as well as basic mechanisms of cellular copper metabolism.

The data presented in these studies provide a model for the molecular basis of WD in patients with the H1069Q allele, in which the mutant protein misfolds and is rapidly degraded by the cell. Based on this framework, a more general model can be proposed in which several factors control the clinical presentation of the disease, including the genotype of the mutation, the percentage of protein that folds correctly and traverses the endoplasmic reticulum, and the effect of the mutation on copper transport and/or trafficking. Such a model may, in part, explain the observations on genotype-phenotype correlation in patients with WD (15), in that gene deletions would present more severely than mutations that decrease protein folding efficiency. In addition, the intragenic clinical heterogeneity that has been observed in WD (38, 39) can be explained by genetic variability in other factors that control protein folding and degradation, analogous to what has been proposed for α_1-antitrypsin deficiency (40). Patient mutations thus may affect protein function both directly through mutation of amino acid residues critical for function and indirectly by enhanced degradation of proteins otherwise competent for copper transport. The stable mammalian expression system described in this report will allow for future detailed functional analyses of mutant proteins and careful dissection of these various disease mechanisms.

The ability of the human Wilson protein to rescue the *mottled* phenotype provides compelling evidence that the Wilson protein is functionally interchangeable with the Menkes protein. This observation and previous studies on the expression of the Menkes protein in *S. cerevisiae* supports the hypothesis that the copper-transporting ATPases work through common biochemical mechanisms in the eukaryotic pathway of cellular copper metabolism (17). These data also suggest that the difference in clinical presentation of Menkes and WD patients is the result of the different expression patterns of the Menkes and Wilson proteins, raising the possibility that interventions to induce expression of the Wilson protein early in development in cells normally expressing the Menkes protein may prevent the otherwise irreversible neuronal degeneration observed in Menkes patients. Even minor up-regulation may be clinically sufficient as a relatively low level of Wilson protein expression (Fig. 1*A*, lanes 2 and 3 versus lane 4) was shown in this study to rescue the *mottled* phenotype, and the residual Menkes protein expression proposed for the occipital horn syndrome (41, 42) is sufficient to prevent neurologic defects in affected patients, whose other symptoms can be managed with early copper therapy (43–45).

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