Copper is a trace element essential for normal cell homeostasis. The major physiological role of copper is to serve as a cofactor to a number of key metabolic enzymes. In humans, genetic defects of copper distribution, such as Wilson’s disease, lead to severe pathologies, including neurodegeneration, liver lesions, and behavior abnormalities. Here, we demonstrate that, in addition to its role as a cofactor, copper can regulate important post-translational events such as protein phosphorylation. Specifically, in human cells copper modulates phosphorylation of a key copper transporter, the Wilson’s disease protein (WNDP). Copper-induced phosphorylation of WNDP is rapid, specific, and reversible and correlates with the intracellular location of this copper transporter. WNDP is found to have at least two phosphorylation sites, a basal phosphorylation site and a site modified in response to increased copper concentration. Comparative analysis of WNDP, the WNDP pineal isoform, and WNDP C-terminal truncation mutants revealed that the basal phosphorylation site is located in the C-terminal Ser796–Tyr1384 region of WNDP. The copper-induced phosphorylation appears to require the presence of the functional N-terminal domain of this protein. The novel physiological role of copper as a modulator of protein phosphorylation could be central to understanding how copper transport is regulated in mammalian cells.

All living organisms require copper for growth and development. Copper is an integral cofactor for numerous enzymes that play key roles in various cell processes, including oxidative metabolism, neurotransmitter synthesis, free radical detoxification, and iron uptake (1–3). Either copper deficiency or copper accumulation is deleterious to cells, and the intracellular concentration of copper is tightly controlled. Two homologous proteins, products of the ATP7A (Menkes disease) and ATP7B (Wilson’s disease) genes, are essential for transmembrane transport of copper in mammalian cells (4–6). Mutations in these genes cause severe pathologies in humans associated with either inborn copper deficiency (Menkes disease) or with vast accumulation of copper in tissues (Wilson’s disease). Both Menkes and Wilson’s disease proteins (WNDP) are copper-transporting P-type ATPases that utilize energy of ATP hydrolysis to transport copper from the cytosol across cell membranes (7–9).

Recent studies indicate that copper plays an active role in regulation of its own metabolism, modulating gene transcription, copper uptake, and protein trafficking (10–13). Although the ability of copper to regulate these cellular events is firmly established, the precise molecular mechanisms through which copper acts are poorly understood. In this study we demonstrate that in human cells changes in copper concentration are “translated” into alterations of the phosphorylation levels for at least one protein target, WNDP. Phosphorylation of WNDP in response to copper is likely to represent a regulatory event, because it is rapid, specific, and reversible and has association with another cellular process, the subcellular redistribution of WNDP. We conclude that protein phosphorylation could be one of the molecular mechanisms by which copper regulates its own metabolism.

EXPERIMENTAL PROCEDURES

Treatment of Cells with Various Metals—Human hepatoma (HepG2) and hepatocarcinoma (Hep3B) cells were grown in minimum essential medium (MEM; Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 0.1 mM MEM nonessential amino acids, and 1 mM sodium pyruvate (basal medium) in a 37 °C, 5% CO2/95% air incubator. At 75–80% confluency, the growth medium was replaced with fresh medium, to which chloride salts of copper or other metals were added at various concentrations (see the figure legends and “Results”). CuCl2, ZnCl2, CoCl2, and CdCl2 were added directly to the medium to the desired concentration (0.5–75 μM); iron was added as a complex with nitrilotriacetic acid. Following 1–12 h of incubation the medium was removed and cells were rinsed twice with PBS (10 mM Na2PO4, 1.76 mM KH2PO4, 3 mM KCl, 137 mM NaCl, pH 7.4) and then collected using a rubber policeman.

To test the reversibility of the copper-induced modification of the WNDP in vivo, cells grown in the basal medium were treated with 5 μM copper for 1 h and then the medium was removed. Cells were either stored at −20 °C (control) or rinsed and then incubated in the basal medium containing 15 μg/ml cycloheximide for 0.5–5 h. Following incubations, cells were collected in 1 ml of PBS containing 1 mM sodium vanadate, centrifuged at 14,000 × g for 30 min at 8 °C, and solubilized with Laemmli sample buffer (1:1 (v/v) of 5% urea:10% SDS:0.5% Tris, pH 6.8) containing 1% β-mercaptoethanol. The lysates were separated by 7.5% Laemmli gel (14) using full-size gel plates (15 cm × 15 cm) and the extended time protocol (25 mA for 8 h or 75 mA for 16–18 h). These conditions were necessary to resolve differences in protein mobility, as shown in Figs. 1, 2, and 5. Minigelis, used for analysis of trans-P incorporation (see below), do not resolve well these mobility differences.

To prepare the microsomal membrane fraction, cells were homogenized in MHTB buffer (0.2 mM mannitol, 0.05 mM sucrose, 1 mM EDTA, 10 mM KCl, 10 mM HEPES, pH 7.5) containing 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride in a tight-fitting Dounce glass homogenizer. Homogenates were centrifuged for 10 min at 500 × g, pellets were discarded, and supernatants were centrifuged for 30 min at 20,000 × g at 100,000 × g for 60 min.

Received for publication, March 7, 2001, and in revised form, July 10, 2001. Published, JBC Papers in Press, July 26, 2001, DOI 10.1074/jbc.M102055200

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4°C (Beckman GS-15R). Pelleted membranes were resuspended in the MHTB buffer, protein concentration was determined by the Lowry method (15), and 25–30 μg were loaded on a full-size 7.5% Laemmli gel. Following electrophoretic separation, proteins were transferred onto polyvinylidene difluoride membrane as described earlier (16), and WNDP was detected using antibody a-ABD at 1:15,000 dilution.

Metabolic Labeling with Inorganic Phosphate—Hep3B cells at 75–80% confluency were incubated in phosphate-deficient MEM (ICN) supplemented with 2 mM glutamine for 4 h at 37°C, 5% CO2. Then, [32P]Inorganic phosphate (0.2 μCi/ml) was added to the medium, and cells were incubated for another 3 h in the presence or absence of 5–10 μM copper added simultaneously with P. Then, the radioactive medium was removed, cells were rinsed with PBS, and membrane fractions were prepared as described above.

Immunoprecipitation—The Ab a-ABD employed for these studies works best with the fully denatured WNDP. Consequently, to achieve efficient immunoprecipitation of WNDP, the metabolically labeled cells were first lysed with sample buffer containing 2.7 M urea, 3.3% SDS, and 1% β-mercaptoethanol, and the lysate proteins were separated by a 7.5% Laemmli gel. The portion of the gel containing the WNDP molecular mass from 130 to 200 kDa was cut out, and proteins were eluted overnight with H2O. The following mixture (1/10 of the eluate volume) was added to the eluted proteins: 10× PBS, 10% milk, 1% Tween 20 and then a-ABD was added at 1:1000 dilution. The eluted proteins were incubated with the Ab for about 30 min at room temperature and then immobilized protein G (Pierce) was added, and the binding reaction was allowed to proceed for 2 h at room temperature. Proteins bound to the immobilized protein G were pelleted by centrifugation and washed for 15 min with TBS, 0.1% Tween three or four times. The radioactivity associated with protein G was monitored using Cherenkov radioactive counting (Beckman) until it reached the steady level.

The [32P]-labeled protein was eluted from protein G by incubation at 50°C for 10 min with Laemmli sample buffer and then separated by 7.5% Laemmli gel. Proteins were then transferred onto polyvinylidene difluoride membrane, and the membrane was air-dried and exposed to either Kodak BioMax MS film or to the CS Bio-Rad molecular imaging screen. The intensity of the [32P] labeling was quantified using Molecular dynamics (Molecular Dynamics, Inc.) at 37°C, 5% CO2. When 50–70% confluent, cells were rinsed once with PBS and treated, for 45 min under growth conditions, with DNA/DEAE-dextran solution (2 μg of plasmid DNA, 250 μg of DEAE-dextran; Amersham Pharmacia Biotech) per 1 ml of PBS. The cells were then incubated in growth medium containing 51 μg/ml of chloroquine (ICN) for 3 h under growth conditions. Next, the cells were treated with 10 μm copper for 2 h and then incubated with PBS. Growth medium was added, and the cells were grown under normal conditions for 24–36 h. Prior to copper treatment and metabolic labeling the growth medium was replaced by phosphate-free Dulbecco’s modified Eagle’s medium (ICN), and cells were grown for an additional 4–12 h. Copper treatment, metabolic labeling, and immunoprecipitation studies were performed as described above for hepatocytes. The preliminary experiments with the G91D mutation revealed that this mutation decreases the expression level of the protein. Therefore, to compare the phosphorylation of the WT and G91D mutant the amounts of WNDP in both cell lysates were estimated before immunoprecipitation. Then, the lysates containing equal amounts of WNDP WT and Gly → Asp proteins were taken into the immunoprecipitation reaction; the difference in the lysate volume was compensated by the lysate from mock-transfected cells. To ensure that the larger volume of lysate does not lead to appearance of nonspecific bands, the corresponding volume of mock-transfected cell lysate was utilized as a control.

RESULTS

Changes of Concentration in the Cell Medium Lead to Reversible Post-translational Modification of WNDP—The starting point of this work was our observation that treatment of cultured human hepatocytes with increasing concentrations of copper induced a change in the electrophoretic mobility of WNDP (Fig. 1A). The upward shift of the WNDP band, detected by Western blot, was small and corresponded to the increase in the WNDP molecular mass of no more than 1–2 kDa. Because copper binding to the recombinant metal-binding domain of WNDP does not induce change in the electrophoretic mobility of the protein (data not shown), we hypothesized that the observed shift was due to copper-induced intracellular modification of WNDP with a small molecular moiety.

A partial change in the WNDP mobility was observed as early as 5 min after exposure to copper (the shortest time interval tested), and the WNDP band was fully shifted up following 30–45 min incubations, as well as after 2-, 3-, and 4-h treatments (data not shown). After 12 h or overnight incubation, the WNDP immunostaining pattern was slightly different. In addition to the WNDP band with modified mobility, protein with original mobility was also present (Fig. 1B), suggesting that chronic copper treatment led to a steady-state equilibrium between the modified and non-modified WNDP forms.

Remarkably, modification of WNDP in response to copper was induced with as little as 1–2 μM copper added to the growth medium (Fig. 1B), pointing to the presence in a cell of a sensitive copper detector. At concentrations of 75 μM and higher, copper becomes inhibitory for cell growth, consequently these high concentrations have not been used in further experiments.
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Copper-induced Modification of WNDP Is Specific and Reversible—Treatment of cells with other metals, such as cobalt, cadmium, iron, and zinc, at concentrations up to 100 μM had no effect on the WNDP mobility (shown in Fig. 1C) or copper-chelator bicinchoninic acid (BCA). The samples were separated by 7.5% Laemmli gel (14) using full-size gel plates (15 cm × 15 cm) and the extended time protocol (25 mA for 8 h or 75 mV for 16–18 h). For this analysis, 10–20% of total lysates obtained from cells grown in 10-cm Petri dishes were utilized. The dilution of a-ABD is 1:20,000.

The copper-induced modification of WNDP is reversible; substitution of the copper-containing medium with the basal medium results in time-dependent changes in the electrophoretic mobility of WNDP opposite to the changes induced by copper. In contrast to rapid modification of WNDP in response to copper, the reverse process takes about 4–5 h to complete (Fig. 2A); in these experiments the protein synthesis inhibitor cycloheximide was added to ensure that the appearance of the band with original mobility was not due to new protein synthesis. These data let us to conclude that within a cell endogenous WNDP responds to changes in copper concentration through reversible post-translational modification.

Hyperphosphorylation of WNDP in response to copper could either be increased phosphorylation of a single site or because of modification of a new site. The 2-fold increase in the [32P] incorporation of WNDP in response to copper could be due to the copper-induced modification of WNDP mobility. The copper-induced modification of WNDP was detected not only in two different hepatic cell lines, Hep3B and HepG2 (not shown), but also in primary retinal epithelium cells (Fig. 1A), suggesting that the observed effect is likely to represent a general phenomenon.

Copper-induced Modification of WNDP Is Sensitive to Reversible—Copper-treated cells and subjected to various treatments. Incubation of WNDP was copper-specific. Significantly, the copper-induced shift in the electrophoretic mobility of WNDP was detected not only in two different hepatic cell lines, Hep3B and HepG2 (not shown), but also in primary retinal epithelium cells (Fig. 1A), suggesting that the observed effect is likely to represent a general phenomenon.

The copper-induced modification of WNDP was confirmed in experiments with 5 μM copper for 1 h. Then cells were transferred into the basal medium containing cycloheximide (15 μg/ml) and incubated for various periods of time (chase in hours). Following cell lysis, the mobility of WNDP was analyzed by gel electrophoresis and Western blot, as described in the legend for Fig. 1. B, microsomal membranes were isolated from control cells and from cells treated with 5 μM copper for 1 h and then membranes were incubated with or without λ-phosphatase (PPase) as described under “Experimental Procedures.” The mobility of WNDP was compared using immunostaining with the a-ABD, as described in the legend for Fig. 1. Each lane contains 20 μg of membrane protein.

Metal Labeling Revealed Two Different Phosphorylation States for WNDP—Hep3B cells were metabolically labeled with [32P]phosphate (Fig. 3). However, the specific radioactivity of the copper-treated WNDP was consistently 2–2.5-fold higher than that of the control protein confirming that copper specifically stimulates phosphorylation of WNDP.

**Fig. 1.** Treatment of hepatic (Hep3B) and retinal (RPE) cells with copper leads to specific and distinct change in the electrophoretic mobility of WNDP. A, short (1-h) treatment with 5 μM copper results in a quantitative upward shift of the WNDP band on a Western blot. B, prolonged treatment (12-h) of Hep3B cells with various copper concentrations leads to a steady-state equilibrium between the non-modified and modified WNDP products. C, the effect of copper (Cu) on WNDP is metal-specific. Shown are the results with 10 μM of each metal or copper chelator bicinchoninic acid (BCA). The samples were separated by 7.5% Laemmli gel (14) using full-size gel plates (15 cm × 15 cm) and the extended time protocol (25 mA for 8 h or 75 mV for 16–18 h). For this analysis, 10–20% of total lysates obtained from cells grown in 10-cm Petri dishes were utilized. The dilution of a-ABD is 1:20,000.

**Fig. 2.** The copper-induced modification of WNDP can be reversed in vivo and in vitro. A, Hep3B cells were incubated in the absence (C) or presence (Cu) of 5 μM copper for 1 h. Then cells were transferred into the basal medium containing cycloheximide (15 μg/ml) and incubated for various periods of time (chase in hours). Following cell lysis, the mobility of WNDP was analyzed by gel electrophoresis and Western blot, as described in the legend for Fig. 1. B, microsomal membranes were isolated from control cells and from cells treated with 5 μM copper for 1 h and then membranes were incubated with or without λ-phosphatase (PPase) as described under “Experimental Procedures.” The mobility of WNDP was compared using immunostaining with the a-ABD, as described in the legend for Fig. 1. Each lane contains 20 μg of membrane protein.
concentrations and can be dephosphorylated with \( \lambda \)-phosphatase.

**Correlation between the Level of Phosphorylation and Intra-cellular Distribution of WNDP**—It has been reported recently that addition of copper to HepG2 cells leads to the intracellular redistribution of WNDP from the trans-Golgi network (TGN) to a vesicular compartment (the precise nature of this compartment remains to be characterized) (17, 18). Therefore we tested whether the copper-dependent change in the WNDP intracellular pattern can be observed in Hep3B cells, which were used primarily for this study, and whether there is a link between the level of phosphorylation of WNDP and its subcellular location. The intracellular distribution and phosphorylation of WNDP were analyzed (i) under basal conditions, (ii) following the 1-h treatment with 10 \( \mu \)M copper, and (iii) following copper treatment and then a 5-h chase in the basal medium. For confocal microscopy experiments, the Hep3B cells were immunostained with the affinity-purified anti-WNDP antibody a-ABD as described under “Experimental Procedures.”

![Fig. 3. Metabolic labeling with inorganic phosphate revealed different phosphorylated states for WNDP.](http://www.jbc.org/)

Hepatic cells grown in 10-cm dishes were metabolically labeled with \([^{32}P]\)inorganic phosphate in the absence (−) or presence (+) of copper as described under “Experimental Procedures.” WNDP was immunoprecipitated and the amount of protein (A) and the radioactivity incorporated into WNDP (B) were compared by densitometry of the WNDP bands (right panels). The lack of mobility shift in this experiment is because of the use of a minigel and shorter time of separation.

![Fig. 4. Basal and copper-induced phosphorylation have different sensitivity to \( \lambda \)-phosphatase.](http://www.jbc.org/)

Microsomal membranes were prepared from the control (−Cu) and copper-treated (+Cu) Hep3B cells and then 20 \( \mu \)g of membrane protein were treated with or without \( \lambda \)-phosphatase as described under “Experimental Procedures.” The amount of radioactivity associated with WNDP was determined by counting the radioactivity associated with the WNDP immunoprecipitates or by densitometry of the WNDP bands (shown). Both methods produced similar results.

![Fig. 5. Correlation between copper-induced phosphorylation of WNDP and its intracellular localization.](http://www.jbc.org/)

The phosphorylation (A) and the intracellular localization (B) of WNDP were analyzed (i) under basal conditions, (ii) following the 1-h treatment with 10 \( \mu \)M copper, and (iii) following copper treatment and then a 5-h chase in the basal medium (Fig. 5, A and B).

As shown in Fig. 5, in the basal medium, where WNDP has a basal level of phosphorylation, the immunostaining of WNDP produced an asymmetric perinuclear staining, consistent with the reported TGN localization of the full-length WNDP (17). Addition of copper to the medium leads to hyperphosphorylation of WNDP and to small but consistent changes in the WNDP pattern. The intensity of the asymmetric staining decreases, and the pattern becomes more uniform, with a vesicle-like staining surrounding the nucleus. Importantly, dephosphorylation of WNDP (Fig. 5, Chase) coincides with re-appearance of the asymmetrical TGN-like perinuclear staining, suggesting that the copper-regulated phosphorylation of WNDP is somehow linked to its subcellular localization.
Mapping the WNDP Regions Important for Basal and Regulated Phosphorylation—Analysis of the WNDP amino acid sequence revealed over 50 potential phosphorylation sites. To identify the regions of WNDP important for its basal and induced phosphorylation the following experiments were carried out. First, two C-terminal truncation mutants of WNDP were generated, WNDP1424 lacking the last 40 amino acid residues and WNDP1384 lacking almost the entire C-terminal tail. The C-terminal truncation mutants and the full-length WNDP were transiently expressed in COS-7 cells and metabolically labeled with 32P inorganic phosphate. The expression of the truncation mutants was comparable with the expression of the full-length WNDP, and both mutants became phosphorylated in the basal medium (Fig. 6A), indicating that most of the C-terminal tail of WNDP was not important for basal phosphorylation.

Second, we compared phosphorylation of the full-length WNDP and PINA (kindly provided by Dr. J. Borjigin). PINA is a product of alternative translation of the WNDP (ATP7B) gene, which lacks the N-terminal copper-binding domain and the first two transmembrane hairpins (19). Fig. 6B illustrates that in the basal medium PINA is phosphorylated similarly to WNDP indicating that the phosphorylation site is located in the C-terminal Me738–Ser1384 region, common for WNDP and PINA.

The quantitative analysis of copper-induced phosphorylation in COS-7 cells was complicated by variability of the response. Although endogenous WNDP, which is present in low quantities in COS-7 cells, was hyperphosphorylated in response to copper (Fig. 7), transiently expressed WNDP did not always show the expected copper-dependent increase of phosphorylation. Because such variability was not observed for phosphorylation of endogenous WNDP, we attributed the inconsistent results to protein overexpression, a hypothesis that we are currently testing. At the same time, measurements of 32P incorporation into PINA reproducibly showed lack of copper-dependent phosphorylation (Fig. 6B), suggesting that the N-terminal domain, which is missing in PINA, could be important for the copper-induced response.

This conclusion was supported by analysis of the full-length WNDP containing the G591D substitution in its N-terminal domain. Earlier, Hamza et al. (20) demonstrated that the G591D mutant had normal intracellular localization but did not interact with the intracellular copper donor HA[H1 and therefore was unlikely to bind copper (20). As shown in Fig. 7, the ability of the G591D mutant to become phosphorylated was unaffected by the mutation, whereas the response to copper was impaired. These results further suggest that the proper folding and/or function of the N-terminal domain could play an important role for the copper-dependent phosphorylation of WNDP.

FIG. 6. The C-terminal tail and the N-terminal domain of WNDP are not essential for its basal phosphorylation. A, the mutants lacking the 40 C-terminal amino acid residues (1424) or 80 C-terminal amino acid residues (1384) were expressed in COS-7 cells at the level similar to the full-length WNDP (WT) treated without (−Cu) or with copper (+Cu), and the ability of these proteins to become phosphorylated was tested by measuring the incorporation of 32P. B, phosphorylation of PINA in the absence and presence of copper was compared with phosphorylation of WNDP under the same conditions. The samples were analyzed using minigels as in Fig. 3.

DISCUSSION

The important metabolic role of copper became increasingly appreciated in recent years. Accumulation of copper in tissues leads to severe pathologies in humans; however the precise molecular consequences of fluctuations in copper concentrations in a cell remains unknown. Here we demonstrate that copper can regulate phosphorylation of protein(s) directly involved in cellular copper homeostasis. Specifically, we show that in a cell the Wilson’s disease protein, a human copper-transporting ATPase, is phosphorylated and that increased copper leads to formation of a “hyperphosphorylated” WNDP. The hyperphosphorylation is removed from WNDP when cells are transferred into the basal medium (see Figs. 2 and 5), thus providing a direct demonstration of the intracellular copper-sensory mechanism at work. It is significant that copper-stimulated phosphorylation is observed at concentrations that do not exceed physiological concentrations of copper in human serum (10–20 μM) and is detected in different cell types.

Protein phosphorylation is a widespread phenomenon playing a key role in the regulation of numerous cellular events contributing to cell growth, development, homeostasis, and cell death. To the best of our knowledge, this is the first report demonstrating that WNDP in a cell is phosphorylated. More importantly, the induced modification of WNDP is the first example of protein phosphorylation specifically regulated by copper. It could be caused by binding of copper to the metal-binding domain of WNDP with concomitant conformational change exposing a specific site for phosphorylation by a kinase (see also below). Alternatively, the copper-dependent phosphorylation could be because of a previously uncharacterized stimulating effect of copper on known kinase(s) or stimulation of a yet uncharacterized copper-dependent kinase. If this later scenario is correct, and there is a kinase-phosphatase system responsive to changes in copper concentration, then such a system may play a profound role in the response of neuronal and hepatic cells to the conditions of copper overload typical for Wilson’s disease.

A stimulatory effect of high copper concentrations on mitogen-activated protein kinase was reported for BEAS cells treated with various metals (21); however the effect of copper on mitogen-activated protein kinase phosphorylation was small and not specific for this metal, in contrast to the highly specific effect of copper reported here. Our experiments on treating cells with various kinase inhibitors demonstrated that H89 (inhibitor of protein kinase A) and SB 203580 (mitogen-activated protein kinase homologue inhibitor) had no effect on copper-induced phosphorylation of WNDP. On the other hand, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (an inhibitor of casein kinase II) partially prevented the phosphorylation (data...
not shown), suggesting that casein kinase II or a casein kinase II-like molecule could be involved in copper-induced phosphorylation of WNDP.

The apparent presence of two separate sites for basal and copper-dependent phosphorylation in the WNDP molecule is particularly interesting. It is suggested by a quantitative shift in the electrophoretic mobility of the hyperphosphorylated WNDP and by different sensitivity of basal and induced phosphorylation to λ-phosphatase. The different effect of the N-terminal domain alterations on the basal and copper-dependent phosphorylation also agrees with the view that there are at least two distinct phosphorylation sites in WNDP. It seems likely that the basal and copper-induced phosphorylations play distinct roles in regulation of WNDP, possibly controlling the intracellular targeting of the protein and overall transport activity of WNDP or modulating the rate of copper release from the N-terminal domain, etc.

Our studies offer the first hint to the possible role of copper-induced phosphorylation. It has been recently reported that copper regulates the intracellular localization of WNDP, stimulating its redistribution from TGN into the vesicular compartment (17, 18). The copper-dependent trafficking of WNDP is an event that apparently determines the rate of copper transport across the membrane by altering the number of transporters present in the membrane. Thus, it is particularly interesting that copper-induced phosphorylation of WNDP coincides with the re-distribution of this protein from TGN into the vesicular compartment, whereas dephosphorylation in response to copper withdrawal is accompanied by the return of WNDP to TGN (Fig. 5). It is tempting to speculate that these two phenomena are linked and that the WNDP trafficking is regulated by copper via reversible copper-dependent phosphorylation. This idea is also supported by close correlation between the concentration and time dependence of the induced phosphorylation observed in our studies and previously reported concentration and time dependence of the copper-induced trafficking of WNDP (18). Alternatively, it is possible that copper-induced phosphorylation takes place only in the vesicular compartment, and upon return to TGN, WNDP became dephosphorylated. Future kinetic and mutagenesis studies will determine which of these hypotheses are correct.

It seems very likely that the N-terminal domain of WNDP plays an important role in the copper-dependent phosphorylation of this protein. The N-terminal domain of WNDP binds copper specifically (22, 23); therefore copper binding to this domain could be the first step triggering the phosphorylation event. Our recent studies revealed that copper binding to the N-terminal domain of WNDP decreases the interactions between the N-terminal domain and the ATP-binding domain of this protein (24). The domain dissociation may therefore expose new sites on either domain, making them available for phosphorylation.

In summary, we demonstrated that in human cells copper could control phosphorylation of distinct protein targets, specifically and in a reversible fashion. This novel physiological function of copper can be central to understanding the mechanisms by which copper transport is regulated in mammalian cells.

Acknowledgments—We thank Dr. Ted Acott for providing primary retinal epithelial cells, Dr. John Denu for helpful discussions and for the generous gift of several phosphatases, Dr. J. Borjigin for providing the PINA construct, Dr. I. Hamza and Dr. J. Gitlin for the G591D mutant of WNDP, and Dr. C. Gatto and Dr. J. H. Kaplan for useful comments and suggestions during manuscript preparation. We are grateful for use of the confocal microscopy facilities at the laboratory of Dr. Susan Amara.

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J. Biol. Chem. 2001, 276:36289-36294.
doi: 10.1074/jbc.M102055200 originally published online July 26, 2001

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