Copper and Zinc Ions Differentially Block Asialoglycoprotein Receptor-mediated Endocytosis in Isolated Rat Hepatocytes*

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ASGP receptors on hepatocytes lose endocytic and ligand binding activity when hepatocytes are exposed to iron ions. Here, we report the effects of zinc and copper ions on the endocytic and ligand binding activity of asialoglycoprotein receptors on isolated rat hepatocytes. Treatment of cells at 37 °C for 2 h with ZnCl₂ (0–220 μM) or CuCl₂ (0–225 μM) reversibly blocked sustained endocytosis of ¹²⁵I-asialoorosomucoid by up to 93% (t₅₀ = 62 min) and 99% (t₅₀ = 54 min), respectively. Cells remained viable during such treatments. Zinc- and copper-treated cells lost ~50% of their surface asialoglycoprotein receptor ligand binding activity; zinc-treated cells accumulated inactive asialoglycoprotein receptors intracellularly, whereas copper-treated cells accumulated inactive receptors on their surfaces. Cells treated at 4 °C with metal did not lose surface asialoglycoprotein receptor activity. Exposure of cells to copper ions, but not to zinc ions, blocked internalization of prebound ¹²⁵I-asialoorosomucoid, but degrada-

tion of internalized ligand and pinocytosis of the fluid-phase marker Lucifer Yellow were not blocked by metal treatment. Zinc ions reduced diferric transferrin binding and endocytosis on hepatocytes by ~33%; copper ions had no inhibitory effects. These findings are the first demonstration of a specific inhibition of receptor-mediated endocytosis by non-iron transition metals.

Cells require transition metals for a variety of crucial cellular processes, but unregulated accumulation of these metals, such as iron and copper ions, promotes damage to membrane lipids, proteins, and nucleic acids (1, 2). As a result, eukaryotic cells acquire nutrient metal ions by highly regulated mechanisms that also guard against overaccumulation of these metals, sequester metal ions when they are present in overabundance, and ameliorate the toxic effects of metals on cellular macromolecules (for reviews, see Refs. 3–5).

We have been studying the hepatic metabolism of the iron-binding protein lactoferrin and have found that rat hepatocytes take up lactoferrin via the rat hepatic lectin (RHL)¹ subunit of the ASGP receptor. Unlike other characterized ligands for the ASGP receptor, lactoferrin binds at or near RHL1’s carbohydrate recognition domain, yet in a carbohydrate-independent manner (6, 7). We have also found that the iron status of hepatocytes alters the manner in which hepatocytes bind and internalize lactoferrin. Iron loading of hepatocytes by incubation of cells with ferric ammonium citrate increases lactoferrin binding and endocytosis. This iron ion-dependent increase in lactoferrin accumulation, however, is accomplished by non-RHL1 binding sites that are reversibly expressed on cells in an iron-dependent manner reminiscent of iron-dependent regulation of ferritin (8, 9). In contrast, the endocytic activity of the ASGP receptor is dramatically inhibited by hepatocyte iron loading (8, 9). In addition, iron-replete hepatocytes accumulate up to half of their ASGP receptors in an inactive form, and these various effects correspond to the appearance of cystinyl-linked ASGP receptor RHL1 subunits (9). These findings represent a heretofore-unrecognized relationship between the ASGP receptor system and the iron status of hepatocytes.

Iron belongs to the borderline class of transition metals, which also includes Mn²⁺, Cu²⁺, Ni²⁺, Cu²⁺, and Zn²⁺ (10). In general, borderline transition metals interact readily with organic ligands and are required by cells in trace amounts to carry out structural, catalytic, or redox activities. Because iron and copper ions are both able to form disulfide-bonded oligomers of RHL1 (9), we speculated that the iron-dependent inhibition of ASGP receptor function was related to the redox properties of iron ions. To address this matter, we initiated a study to examine the effects of other borderline transition metals (Mn²⁺, Cu²⁺, and Zn²⁺) with various redox capacities on ASGP receptor endocytic activity. Like iron ions, both manganese and copper ions can mediate intracellular redox reactions whereas zinc ions are not redox active. Here, we report that copper and zinc ions, but not manganese ions, dramatically block ASGP receptor-mediated endocytosis and receptor ligand binding activity. Moreover, zinc, but not copper, partially blocked internalization of transferrin by hepatocytes, but neither metal blocked intracellular processing of internalized ligand nor pinocytosis. Our findings are the first demonstration of a specific inhibition of receptor-mediated endocytosis by non-iron transition metals.

**Experimental Procedures**

*Materials—Human asporosomucoid, human holotransferrin, neuraminidase (Clostridium perfringens), Lucifer Yellow (dipotassium salt), and BSA (fraction V) were purchased from Sigma. IODOGEN, IgG purification kit, and BCA protein assay reagent were obtained from Pierce.*

Collagenase (type D) was from Roche Molecular Biochemicals. Digitonin was obtained from Eastman Kodak. N,N-dimethylethanolamine was purchased from Sigma. IODOGEN, IgG purification kit, and BCA protein assay reagent were obtained from Pierce.

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Hepatocyte Preparation—Male Sprague-Dawley rats (100–350 g, Harlan Sprague, San Diego, CA) were fed standard laboratory chow and...
Copper and Zinc Ions Block ASGP Receptor Dynamics

water ad libitum. The copper and zinc content of the chow (Purina Rodent Laboratory Chow, formula 5001) were 13 and 70 ppm, respectively, as assayed by the manufacturer. Hepatocytes were prepared by a modification of a collagenase perfusion procedure (11) as described previously (12). Cells were kept at approximately 30 °C during the filtrations and were described as 100% viable before experiments. Final concentrations of copper and zinc ions in hepatocytes incubated in ice-cold BME-BSA were 70–110 dpm/fmol. Hepatocytes in BME-BSA were incubated with or without CuCl2 or ZnCl2 for 2 h at 37 °C. To determine surface binding of 125I-transferrin by hepatocytes, cells were chilled on ice and incubated 2 h with 125I-transferrin (2 μg/ml) in BME at 4 °C. The radioactive mixture was removed, and the cells were rinsed four times with cold Buffer A. The cells and radiolabeled tissue were solubilized by incubating the cells in 2 ml of Buffer A containing 2% (v/v) Triton X-100 at 37 °C for 2 h. Detergent-released 125I-radioactivity was transferred to clean tubes and assayed by γ spectrometry. To determine 125I-transferrin endocytosis by hepatocytes, cells in BME-BSA were supplemented with 125I-transferrin (2 μg/ml) and incubated at 37 °C for 60 min, after which the cells were chilled on ice. Radioactive media were removed, and the cells were assayed for total bound 125I-transferrin as described above. To determine the amount of internal 125I-transferrin, rinsed cells at 4 °C were stripped of surface-bound 125I-transferrin by a 30 s exposure to 0.25 M acetic acid, 0.5 M NaCl, after which an equal volume of ice-cold 0.5 M NaHPO4 was added to bring the pH to 7.5. Acid-released radioactivity was aspirated off the cells, and the cells were rinsed an additional three times with cold Buffer A. Acid-resistant radioactivity, deemed intracellular, was assayed following a 5 min solubilization as described above.

**RESULTS**

Zinc and Copper Block Sustained ASGP Receptor-dependent Endocytosis—In the first series of experiments, we examined the effects of MnCl2, ZnCl2, or CuCl2 on sustained endocytosis (binding and internalization) of 125I-ASOR by hepatocytes. Cells exposed to MnCl2 (0–220 μM) exhibited no alteration in sustained endocytosis of 125I-ASOR (data not shown). In contrast, continuous internalization of 125I-ASOR by cells incubated with 0–220 μM ZnCl2 (Fig. 1A) or 0–225 μM CuCl2 (Fig. 1B) diminished up to at least 94%. Maximal inhibition of endocytosis (Fig. 1B, inset) was observed at 39 μM CuCl2 and 53 μM ZnCl2 although significant inhibition exerted by ZnCl2 occurred only at concentrations >20 μM. Inhibition of continuous 125I-ASOR endocytosis by ZnCl2 (220 μM) or CuCl2 (75 μM) was time-dependent (Fig. 2). The first-order half-time rates for loss of 125I-ASOR internalization were 62 min for zinc ions and 54 min for copper ions (Fig. 2, A and B, insets). Overall, the loss of 125I-ASOR binding and uptake followed similar dose-response curves (Fig. 1) and kinetics (Fig. 2). Regardless of the treatment, cell viability was not altered, as determined by trypan blue exclusion by control and metal-treated cells (data not shown). We also found that the effects of copper and zinc ions on continuous uptake of 125I-ASOR were additive (Fig. 3). Binding and internalization of 125I-ASOR were more greatly inhibited when cells were treated with a combination of ZnCl2 (220 μM) and CuCl2 (75 μM) (91% and 95% for zinc and copper, respectively) than when treated with either metal alone. We also found that the effects of zinc and copper on ASOR endocytosis were not ameliorated by co-incubation with the general reduttent arsobacte (Table 1). Notably, asborate (0.1 mM) enhanced the copper-dependent inhibition of ASOR binding and internalization compared with cells treated with copper ions alone.

The inhibition of ASGP receptor-dependent endocytosis by metal treatment was reversible. Cells treated with ZnCl2 or

Lucifer Yellow fluorescence (excitation at 430 nm, emission at 540 nm) using a Fluoromax-2 spectrofluorimeter (Jobin Yvon Spex, Instruments S.A., Edinbour, NJ).

125I-Transferrin Binding and Endocytosis Assay—Adult rat hepatocytes were plated on unmodified tissue culture plates and incubated for 20 h at 37 °C in Minimum Essential Medium (MEM) before experiments. Cells were incubated in BME-BSA (no serum present) with or without CuCl2 or ZnCl2 for 2 h at 37 °C. To determine surface binding of 125I-transferrin by hepatocytes, cells were chilled on ice and incubated 2 h with 125I-transferrin (2 μg/ml) in BME at 4 °C. The radioactive mixture was removed, and the cells were rinsed four times with cold Buffer A. The cells and radiolabeled tissue were solubilized by incubating the cells in 2 ml of Buffer A containing 2% (v/v) Triton X-100 at 37 °C for 2 h. Detergent-released 125I-radioactivity was transferred to clean tubes and assayed by γ spectrometry. To determine 125I-transferrin endocytosis by hepatocytes, cells in BME-BSA were supplemented with 125I-transferrin (2 μg/ml) and incubated at 37 °C for 60 min, after which the cells were chilled on ice. Radioactive media were removed, and the cells were assayed for total bound 125I-transferrin as described above. To determine the amount of internal 125I-transferrin, rinsed cells at 4 °C were stripped of surface-bound 125I-transferrin by a 30 s exposure to 0.25 M acetic acid, 0.5 M NaCl, after which an equal volume of ice-cold 0.5 M NaHPO4 was added to bring the pH to 7.5. Acid-released radioactivity was aspirated off the cells, and the cells were rinsed an additional three times with cold Buffer A. Acid-resistant radioactivity, deemed intracellular, was assayed following a 5 min solubilization as described above.

**General Procedures**—Protein was determined by the bicinchoninic acid protein assay procedure using BSA as standard (Pierce). Centrifugation of cell suspensions was at 400 × g for 2 min at 4 °C using a Beckman GS-6R centrifuge equipped with a GH-3.8 rotor (Beckman Instruments, Inc., Fullerton, CA). 125I radioactivity was determined using a Packard Cobra Auto-Gamma counting system (model 5002; Packard Instrument Co., Downers Grove, IL). Spectroscopic measurements were done using a Shimadzu UV-160 spectrophotometer (Kyoto, Japan).

**RESULTS**

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Copper and Zinc Ions Block ASGP Receptor Dynamics

**Fig. 1. Continuous endocytosis as a function of metal concentration.** Hepatocytes (2 × 10^6 cells/ml) were incubated in BME-BSA with and without the designated concentrations of ZnCl₂ (A) or CuCl₂ (B) at 37 °C for 2 h. The cells were supplemented with 125I-ASOR (2 µg/ml), and the incubations were continued at 37 °C for an additional 60 min. The cells were chilled at 4 °C and assayed for total and internalized 125I-ASOR. Symbols reflect the mean of duplicate samples; error bars reflect the standard deviations of the means. Results shown in panels A and B were from experiments done on different days using different hepatocyte and 125I-ASOR preparations. The extent of inhibition (inset) was calculated according to the equation (X_{max} - X_{in})/X_{max} - X_{min} = inhibition at metal concentration C, and X_{max} = levels of 125I-ASOR total bound or internalized in the absence of added metal ions. Symbols represent extent of inhibition of 125I-ASOR binding (surface and intracellular; ○, ■) and internalization (●, ●) in the presence of copper (○, ●) or zinc (○, ●) ions.

CuCl₂ exhibited >60% loss in total (surface and intracellular) bound (Fig. 4A) and internalized (Fig. 4B) 125I-ASOR. When cells were washed free of metals and incubated in the absence of exogenous zinc and copper ions, they recovered their ability to bind and take up 125I-ASOR. These results indicate that the metal-sensitive sites required for endocytosis were either not irreversibly blocked by metal treatment or were readily replaced following de novo synthesis. In either case, these data support the conclusion that the block in endocytosis was not due to a loss of cell viability.

In each of the preceding experiments, zinc- or copper-treated hepatocytes were examined for their ability to take up 125I-ASOR continuously during a 1-h assay period following a 2-h metal treatment period. As a result, inhibition of continuous endocytosis could reflect a block in one or more events including internalization of receptor-ligand complexes at the cell surface, intracellular processing of receptor-ligand complexes, or recycling of ASGP receptors. To determine if zinc or copper ions blocked internalization of receptor-ligand complexes at the cell surface, we examined the ability of metal-treated hepatocytes to internalize 125I-ASOR prebound on the cell surface (Fig. 5). We found that the amount of 125I-ASOR bound to the cells following zinc and copper treatment was reduced by 56% and 35%, respectively, reflecting a loss in the number of active ASGP receptors on the surfaces of metal-treated cells. Zinc-treated cells internalized progressively less 125I-ASOR as a function of ZnCl₂ concentration but the percentage of bound 125I-ASOR that was internalized remained the same (-30%) regardless of the ZnCl₂ concentration (Fig. 5A). In contrast, the percentage of prebound 125I-ASOR internalized by cells was reduced from 33% on control cells to <5% on cells treated with 75 µM CuCl₂ (Fig. 5B). These results indicate that, although both metals reduced the number of active surface ASGP receptors, copper ions, but not zinc ions, blocked the ability of cells to internalize receptor-ligand complexes at the cell surface. These
Hepatocytes (2 × 10^6 cells/ml) in BME-BSA were incubated with or without the designated amounts of ZnCl₂, CuCl₂, or ascorbate for 2 h at 37°C. Cells were chilled at 4°C, washed twice in BME-BSA to remove excess metal, then prebound with ¹²⁵I-ASOR at 4°C for 1 h. After cells were washed free of unbound ¹²⁵I-ASOR, cells were resuspended in fresh BME-BSA with or without ZnCl₂, CuCl₂, or ascorbate (similar to that during the original 2-h incubation) and incubated at 37°C for 15 min. The cells were chilled at 4°C and assayed for total bound and internalized ¹²⁵I-ASOR. Values represent the mean ± standard deviation of duplicate samples. Values as a function of a percentage of untreated control cells are included in parentheses.

### Table I

**Effect of ascorbate, Zn²⁺, and Cu²⁺ treatment on ASGP receptor endocytic activity**

| Metal | [Metal] (µM) | [Ascorbate] (µM) | Zn²⁺ Total | Zn²⁺ Internal | Cu²⁺ Total | Cu²⁺ Internal |
|-------|--------------|-----------------|------------|---------------|------------|---------------|
| Zn²⁺  | 0            | 0.1             | 254 ± 3 (100) | 181 ± 2 (100) | 68 ± 5 (27) | 21 ± 1 (12)   |
|       | 0            | 1.0             | 256 ± 3 (101) | 182 ± 4 (100) | 76 ± 2 (30) | 30 ± 8 (17)   |
|       | 0.3          | 1.0             | 77 ± 2 (30)   | 35 ± 4 (19)   | 60 ± 3 (24) | 26 ± 1 (14)   |
| Cu²⁺  | 75           | 0.1             | 371 ± 10 (100) | 269 ± 3 (100) | 260 ± 1 (70) | 25 ± 6 (9)    |
|       | 0            | 1.0             | 354 ± 2 (95)  | 281 ± 6 (104) | 201 ± 2 (54) | 16 ± 2 (6)    |
|       | 75           | 0.3             | 202 ± 1 (54)  | 11 ± 1 (4)    | 213 ± 1 (57) | 11 ± 1 (4)    |

Activity—The previous experiments (Fig. 5, Table I) showed that copper- and zinc-treated hepatocytes reduced the number of active ASGP receptors on their surfaces. To address this issue more directly, we examined the ligand binding activity of surface and intracellular populations of ASGP receptors on metal-treated hepatocytes. Surface ASGP receptor activity was assayed on intact cells, whereas both surface and intracellular ASGP receptor activity was assayed on digitonin-permeabilized hepatocytes (15, 17). Treatment of cells with ZnCl₂ or CuCl₂ reduced surface binding of ¹²⁵I-ASOR by up to 44% (Fig. 6A and 48% (Fig. 6B), respectively. Permeable metal-treated cells also bound progressively less ¹²⁵I-ASOR than did non-treated control cells. Notably, the absolute molar reduction of ¹²⁵I-ASOR binding to permeable cells was similar to that observed for intact cells, such that the calculated number of active intracellular ASGP receptors was not altered by metal treatment (Fig. 6A and B, closed circles). Thus, both copper and zinc ions induced a loss of surface ASGP receptor activity with no net change in the amount of intracellular receptor activity.

The loss of receptor-ligand binding activity observed in the previous experiment does not necessarily reflect the loss of receptor protein from the cell surface. Thus, we examined the surfaces of metal-treated cells for the presence of immunode-
A RHL1 protein was lost from the cell surface. In contrast, cop-}

tective ASGP receptor protein by assaying their ability to bind

did control cells indicating that RHL1 protein was lost from the cell surface. In contrast, cop-

per-treated cells exhibited no loss of 125I-anti-RHL1 Ig binding (Fig. 6D). Treatment of cells with zinc or copper ions at 4 °C either before or during the 125I-anti-RHL1 Ig binding assay did not alter 125I-anti-RHL1 Ig binding to the cells,2 indicating that copper and zinc ions per se did not interfere with antibody binding to the receptor. Taken together, these data indicate that copper-treated cells accumulated inactive ASGP receptors at the cell surface with no significant change in the distribution of RHL1 subunits between the cell surface and cell interior. Because zinc ion-treated cells lost surface ASGP receptor activity and protein without an increase in intracellular ASGP receptor activity (Fig. 6A), we conclude that zinc-treated cells accumulated inactive ASGP receptors intracellularly.

In other experiments, we found that the metal-induced re-
duction of surface ASGP receptor activity was temperature-de-
pendent. Cells treated with various concentrations of zinc or copper ions at 4 °C showed no loss of surface binding of 125I
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ASOR (Fig. 7A) compared with cells pretreated at 37 °C with either CuCl2 or ZnCl2 alone or in combination (Fig. 7B). These data indicated that metal-induced losses of surface receptor activity results from perturbations in one or more cellular processes rather than direct interference of metals on the abil-
ity of ASGP receptors to bind ligand per se. Notably, significant metal-dependent reductions of surface 125I-ASOR binding were

evident only when cells were exposed to metal ions at or above 22 °C (Fig. 7C), and 125I-ASOR binding was actually higher on samples treated with different levels of CuCl2 than on control cells. In other experiments, we found that the ASGP receptor binding of ASOR was partially reduced for zinc-treated 125I
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ASOR (Table II). When cells were incubated with 125I-ASOR and zinc or copper ions simultaneously at 4 °C, binding of

of 125I-ASOR was partially reduced for zinc-treated 125I
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ASOR compared with non-pretreated 125I-ASOR (Table II). In

About half of the ASGP receptors on isolated rat hepatocytes recycle constitu-
tively, whereas the rest recycle only during endocytosis (18). Reduced surface ASGP receptor activity and ASGP receptor-
dependent endocytosis on metal-treated hepatocytes may re-
fect, in part, an interruption in the return of recycling ASGP receptors back to the cell surface. If metal treatment interferes with ASGP receptor recycling, then one would predict that the loss of surface ASGP receptor activity would be more pro-
nounced if hepatocytes were actively endocytosing ASOR during
treatment with copper or zinc ions. To test for this, hepa-
tocytes were treated with various concentrations of ZnCl2 or

2 D. D. McAbee and X. Jiang, unpublished observations.
of their surface ASOR binding activity. The ASOR-dependent reduction in surface ASGP receptor activity was not enhanced when cells were treated with zinc ions (Fig. 8A). On the other hand, cells treated with 75 μM CuCl2 showed a significant ASOR-dependent reduction in surface ASGP receptor activity (Fig. 8B), suggesting that copper ions, but not zinc ions, par-

| Table II | Effect of Zn2+ and Cu2+ on ASGP receptor ligand-binding activity |
|----------|---------------------------------------------------------------|
| Experiment | Treatment | 125I-ASOR | 125I-ASOR bound |
| 1 | None | None | 274 ± 11 (100) |
| | ZnCl2 | None | 267 ± 4 (97) |
| | CuCl2 | None | 285 ± 2 (104) |
| | ZnCl2 | ZnCl2a | 196 ± 35 (72) |
| | CuCl2 | CuCl2a | 256 ± 11 (93) |
| 2 | None | None | 567 ± 20 (100) |
| | ZnCl2 | None | 531 ± 1 (94) |
| | CuCl2 | None | 583 ± 12 (103) |

* In these cases, 125I-ASOR was preincubated with 220 μM ZnCl2 or 75 μM CuCl2 for 1 h at 22 °C, then chilled and added to hepatocytes for 1 h at 4 °C, and cell-bound radioactivity was determined.

FIG. 8. Effect of endocytic load during metal treatment on surface ASGP receptor activity. Hepatocytes (2 × 10⁶ cells/ml) in BME-BSA were incubated with or without ZnCl2 (A) or CuCl2 (B) in the presence (●) or absence (■) of ASOR (2 μg/ml) at 37 °C for 2 h. The cells were chilled at 4 °C, washed in Buffer B at 4 °C to remove surface-bound ASOR, then assayed for 125I-ASOR binding at 4 °C. Symbols represent means of duplicate samples; error bars reflect standard deviations of the means.
stimulated receptor recycling is not observed.

We also examined the effects of zinc and copper ions on the lysosomal delivery and degradation of internalized 125I-ASOR. In this experiment, hepatocytes were prebound with 125I-ASOR at 4 °C, then incubated at 37 °C. At various times after the temperature shift, the cells were supplemented with metals and the 37 °C incubation was continued for up to 2 h at which time the amounts of cell-associated 125I-ASOR and 125I-degradation products released from the cells were determined (Fig. 9). We found that neither zinc (Fig. 9A) nor copper (Fig. 9B) ions significantly altered the amount of 125I-ASOR degraded by the cells as measured by the release of acid-soluble radioactivity from the cells, even if metals were added just 10 min following the shift to 37 °C. These results indicate that neither zinc nor copper ions perturbs the intracellular dissociation of internalized ASGP receptor-ASOR complexes or the delivery of ASOR to lysosomes and its subsequent degradation.

**Effect of Copper and Zinc on Fluid-phase Pinocytosis and Transferrin Endocytosis**—The preceding results collectively show that zinc and copper ions strongly block ASGP receptor-mediated endocytosis. The question arises as to whether or not the inhibitory effects of these metal ions are specific for ASGP receptors or whether other vesicular uptake pathways are also sensitive to the effects of these metals. In the first series of experiments, we determined if fluid-phase uptake by hepatocytes was altered by copper or zinc ions. It has been shown that clathrin-dependent receptor-mediated endocytosis and pinocytosis can be uncoupled in hepatocytes by K+ depletion or by hyperosmotic treatment (19). In this experiment, isolated rat hepatocytes were treated at 37 °C for 2 h with or without ZnCl2 (225 μM) or CuCl2 (75 μM). After incubation, the cells were assayed for 125I-ASOR endocytosis, to measure ASGP receptor activity, and Lucifer Yellow uptake, to measure fluid-phase pinocytosis. As anticipated, zinc and copper ions inhibited 125I-transferrin internalization at 37 °C (Fig. 11A). Under these conditions, however, Lucifer Yellow uptake was largely unaffected (Fig. 10B). These findings suggest strongly that neither zinc nor copper ions interrupted clathrin-independent fluid phase vesicular transport in hepatocytes.

Finally, we examined the effect of copper or zinc loading on transferrin binding and endocytosis by hepatocytes. We found that copper-treated hepatocytes showed no alteration in 125I-transferrin binding at 4 °C or 125I-transferrin binding and internalization at 37 °C (Fig. 11B) in contrast to 125I-ASOR endocytosis, which was blocked by ~70% (Fig. 11A). Zinc treatment of hepatocytes, however, elicited a 50% reduction in surface 125I-transferrin binding at 4 °C and a 35% reduction in 125I-transferrin internalization at 37 °C (Fig. 11B). We found that preincubation of 125I-transferrin with ZnCl2 did not alter its ability to bind to hepatocytes subsequently.2 These data indicate, therefore, that zinc treatment of hepatocytes partially disrupted their ability to endocytose transferrin.

**DISCUSSION**

**Specificity of Copper and Zinc Inhibition of Endocytosis**—We are interested in understanding the role metalloproteins play in vesicular transport and the sensitivity of membrane dynamics toward metal toxicity. In this study, we have examined the effects of zinc and copper ion exposure on vesicular transport in

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**Fig. 9.** Effect of metal treatment on degradation of internalized 125I-ASOR. Hepatocytes (2 × 10⁶ cells/ml) in BME-BSA were incubated with 125I-ASOR at 4 °C for 1 h, after which they were washed twice in Buffer A to remove unbound 125I-ASOR. Cells were resuspended in fresh BME-BSA and incubated at 37 °C. At the indicated times, cells were supplemented with ZnCl2 (225 μM; A) or CuCl2 (75 μM; B), and the incubation was continued for a total of 2 h. Samples shown at 120 min were not exposed to added metals. The cells were chilled at 4 °C. Cell media were collected and assayed for acid-soluble (■) and acid-precipitable (○) radioactivity. Cells were assayed for associated radioactivity (▲). Symbols represent the means of duplicate samples; error bars reflect the standard deviations of the means. Plotted lines are the best-fit curves calculated by linear regression (y = mx + b) of data points.

**Fig. 10.** Effect of metal treatment on fluid-phase uptake. Hepatocytes (4 × 10⁶ cells/ml) in BME-BSA were incubated in the absence (□) or presence of CuCl2 (■) or ZnCl2 (○) for 2 h at 37 °C. Cell samples were supplemented with 125I-ASOR (2 μg/ml) and Lucifer Yellow (0.2 mg/ml) and incubated an additional 60 min. The cells were chilled on ice and washed twice with cold BME-BSA (10 min/wash). Cells were solubilized in HBS supplemented with 0.05% (v/v) Triton X-100 and BSA (1 mg/ml) for 30 min at 4 °C, after which insoluble debris was sedimented by centrifugation (13,000 × g, 4 °C, 15 min). Supernatants were assayed for 125I-ASOR (A) and Lucifer Yellow (B). Symbols represent the mean of duplicate samples; error bars reflect the standard deviations of means.
isolated rat hepatocytes. Several lines of evidence support the view that the effects of copper and zinc were specific for receptor internalization and recycling and not for vesicular transport in general. First, neither metal blocked significantly the transport to and subsequent degradation of internalized ASOR in lysosomes (Fig. 9), even though both copper and zinc ions severely reduced continuous ASOR endocytosis (Figs. 1–3 and 11). These data suggest that copper and zinc ions under these conditions do not block ASGP receptor/ASOR dissociation and segregation for ASOR en route to lysosomes. Second, copper and zinc ions did not alter appreciably fluid-phase pinocytosis of Lucifer Yellow by hepatocytes under conditions where ASOR endocytosis was severely reduced (Fig. 10). Hepatocytes internalize bulk fluid predominately by a clathrin-independent pathway such that hyperosmotic disruption of clathrin-dependent uptake of ASOR by ASGP receptors has little or no effect on Lucifer Yellow uptake (19). Thus, the molecular machinery that mediates vesicular budding for this clathrin-independent pathway apparently is not susceptible to the effects of copper or zinc ions. Third, transferrin endocytosis in copper-treated hepatocytes was not reduced, as was ASOR endocytosis (Fig. 11). These data suggest that the effects of copper ions on ASGP receptor-dependent uptake were specific for this receptor and not inhibitory for endocytosis in general. In contrast, zinc ions blocked or reduced uptake of ASOR and transferrin by hepatocytes, suggesting the possibility that a mechanism or structural motif necessary for the proper functioning of these two receptors was sensitive to the effects of zinc ions.

Although the effects of copper and zinc ion exposure were relatively specific for ASGP receptor-mediated endocytosis, not all ASGP receptors responded to metal treatment equally. In several experiments, we found that a portion of surface ASOR receptor activity was lost when cells were exposed to copper or zinc ions (Figs. 5–8). In one instance, metal-dependent reductions of surface 125I-ASOR binding were evident only when cells were exposed to metal ions at or above 22 °C (Fig. 7C), and cells treated with copper ions at temperatures below 33 °C frequently expressed more surface ASOR receptor activity than did control cells (Fig. 7C). Copper ion transport in rat cells is temperature-dependent (20), and the amount of copper or zinc ions accumulated by hepatocytes necessary to block endocytosis may be temperature-dependent. Alternatively, it may be that copper and zinc ions exert their effects only on ASGP receptors actively moving along the endocytic pathway. Only a subset of ASGP receptors, termed “state 2” ASGP receptors (21), recycle constitutively. When isolated rat hepatocytes are incubated at temperatures below 37 °C, the surface:intracellular ratio of state 2 ASGP receptors reversibly decreases (22). Accordingly, copper-treated hepatocytes may have consistently expressed higher surface ASGP receptor activity than did control cells (Fig. 7C) because copper ions, by inhibiting ASGP receptor internalization (Fig. 5), blocked the temperature-induced shift of receptors from the cell surface to the cell interior. Even so, it is expected both “state 1” and state 2 receptor classes are sensitive to these metals under conditions when both populations are mediating endocytosis of ligand.

Effect of Transition Metals on ASGP Receptor Activity—Copper and zinc ions induced cells to accumulate a portion of their ASGP receptors in an inactive form (Fig. 6). Hepatocytes depleted of ATP or treated with agents that alkalinize acidic endosomal compartments (e.g. chloroquine, monensin) reversibly accumulate inactive ASGP receptors with no loss of total detectable ASGP receptor protein (22, 23). Weigel and colleagues (24–26) have found a direct correlation between ASGP receptor ligand binding activity and receptor palmitoylation (at Cys35 of RHL1) such that decysylated ASGP receptor subunits do not bind ASOR. In addition, constitutive recycling of ASGP receptors and receptor inactivation/reactivation are not necessarily coupled, as monensin treatment (22) and copper treatment (this report) induce the loss of ligand binding activity of ASGP receptors at the cell surface with little or no loss of immunodetectable receptor protein. Because copper treatment blocks ASGP receptor internalization (Figs. 5 and 8), it is likely that inactive ASGP receptors are accumulating on the surfaces of copper-treated cells. On the other hand, zinc-treated hepatocytes lost both ASGP receptor activity and immunodetectable RHL1 protein from the cell surface (Figs. 5 and 6), and this lost surface ASGP receptor activity was not recovered when permeable cells were assayed for 125I-ASOR binding (Fig. 6). It appears, therefore, that zinc-treated cells accumulated inactive ASGP receptors intracellularly, as zinc treatment alone did not block internalization of receptors from the cell surface (Fig. 5).

Our previous observations, which showed a correlation between iron-dependent inactivation of ASGP receptors and formation of disulfide-bonded RHL1 multimers, suggested the possibility that iron-dependent oxidation of Cys35 could preclude RHL1 acylation and thereby promote receptor inactivation (8, 9). We have found that hepatocytes treated with copper or zinc ions lose ≤90% of reduced glutathione content, suggesting that both metals induce a shift in the oxidative status of the cells. Under these conditions, we frequently (but not always) detect RHL1 dimers formed to a limited extent in hepatocytes treated with copper ions; we have never detected RHL1 multimers on zinc ion-treated cells. Thus, copper and zinc ions may induce ASGP receptor inactivation by disrupting receptor reacylation via a mechanism other than oxidation of Cys35.

Nature of Copper- and Zinc-sensitive Sites—The nature of copper or zinc ion inhibition of ASGP receptor-mediated endocytosis is unclear. Both Cu2+ and Zn2+ are borderline metals and show increased binding strength to organic ligands compared with class A metals (e.g. K+, Na+, Mg2+, and Ca2+) (10). Zinc ions typically function by conferring specific structures to proteins they bind (e.g. zinc-finger proteins), whereas copper ions perform redox reactions in copper ion-bound proteins (e.g. superoxide dismutase). One possible mode of endocytic inter-
ruption is that OH· generated by copper ion-mediated Fenton reaction would oxidatively damage lipids and proteins crucial for normal uptake and recycling of ASGP receptors (1). Evidence against this possibility includes the following. (i) The copper ion inhibition of ASOR binding and endocytosis were largely reversed following incubation of copper-treated cells in the absence of copper ions (Fig. 4). (ii) Copper ions did not alter significantly bulk-phase pinocytosis of Lucifer Yellow or transferrin endocytosis by treated hepatocytes. Because transferrin and ASOR are internalized by the same set of endocytic machinery, inactivation or damage exerted on this machinery by reactive oxygen species would block uptake of both ligands. (iii) We did not observe significant diminishment of cell viability as a result of copper ion treatment. (iv) Exposure of hepatocytes with up to 220 µM MnCl2 had no detectable effect on ASGP receptor-mediated endocytosis. This finding is significant because manganese ions have been shown to facilitate generation of reactive oxygen species (27). Because zinc ions are not redox active and do not mediate production of reactive oxygen species, these findings taken together suggest that reactive oxygen species were not responsible for the inhibitory effects of these metal ions on ASGP receptor-mediated endocytosis.

An alternate hypothesis is that copper and zinc ions bind ASGP receptors and interfere directly with their function. RHL1 does not contain canonical copper or zinc binding domains (e.g. MXM, MXMX, CC, CXC, or CXXC) present in metallothioneins (28) or FYVE-finger proteins (29). Even so, copper and zinc ions likely coordinate with a common set of histidyl and carboxyl groups on the RHL1 cytoplasmic domain, some of which are located near the internalization signal YQDF (amino acids 5–8) (30). Nonetheless, we observed significant differences in the effects of the two metals. First, unlike copper ions, zinc ions did not block internalization of receptor-ligand complexes from the cell surface (Fig. 5). Second, ASOR binding to ASGP receptors was itself partially altered by zinc ions but not by copper ions (Table II). Regardless, the extent of zinc-dependent inhibition of endocytosis and accumulation of inactive ASGP receptors cannot be attributed solely to the direct inhibition of ASOR binding by zinc ions (<30% reduction, Table II). Third, zinc ions, but not copper ions, blocked at least partially transferrin endocytosis (Fig. 11). Fourth, inhibition of ASOR endocytosis required higher zinc ion concentrations compared with copper ions (Figs. 1, 5). Thus, copper and zinc ions may bind to elements of the cytoplasmic tail of ASGP receptor subunits which directly or indirectly interfere with the internalization signal (copper ions) or the trafficking of ASGP receptors (and transferrin receptors in the case of zinc ions) back to the cell surface, thereby, diminishing sustained endocytosis.

Our findings here provide evidence that transition metal toxicity on cells can be highly specific toward the function of membrane proteins. We are currently investigating whether or not iron, copper, and zinc interact with the cytoplasmic tail of RHL1 subunits, and if so, determine the specific amino acids involved in that interaction.

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