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Original Research

Protease sensitivity and redistribution of CD71 and glycophorin A on K562 cells

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Abstract: Transmembrane proteins are delivered to plasma membrane from the endoplasmic reticulum and Golgi complex by vesicular transport along with the cytoskeletal network. Disruption of this process likely affects transmembrane protein expression. K562 cells were digested with Streptomyces griseus protease for different periods of time, and then re-cultured with different cytoskeletal and glycosylation inhibitors. Cell viability and surface expression of transferrin receptor (CD71) and glycophorin A (GPA) were analyzed before and after re-culture by flow cytometry. We found that digestion with protease almost completely removed extracellular CD71 and GPA but their expression recovered to the initial levels after re-culture for 8 h and 24 h, respectively. The microtubule depolymerizer colchicine promoted cell surface recovery of CD71 but inhibited that of GPA; the microtubule stabilizer paclitaxel inhibited cell surface recovery of CD71 but promoted that of GPA; the microfilament depolymerizer cytochalasin D had no effect on cell surface recovery of CD71 and GPA; the microfilament stabilizer phalloidin inhibited cell surface recovery of GPA. The glycosylation inhibitor tunicamycin inhibited the recovery of both CD71 and GPA, and BADGP inhibited the recovery of GPA. These studies show differential sensitivities of surface proteins on K562 cells to proteases, and suggest molecular mechanisms of transmembrane protein transport and cycling.

Key words: Transmembrane protein transport; Protease digestion; Cytoskeleton inhibitors; Glycosylation inhibitors; Flow cytometry.

Introduction

There are six mechanistic classes of proteases: serine proteases, metalloproteases, aspartic proteases, threonine proteases, glutamic acid proteases and cysteine proteases; all catalyze the hydrolytic degradation of proteins or polypeptides into shorter polypeptide fragments or amino acids by splitting the peptide bonds that link amino acid residues (1). Protease from S. griseus (streptomyces) is a mixture of proteases with at least three caseinolytic activities and one aminopeptidase activity, including an extracellular serine protease (2). The caseinolytic enzymes were named as S. griseus Protease A, S. griseus Protease B and S. griseus Trypsin. Serine proteases display a wide range of substrate specificities, which are believed to be mediated by an active site composed of one Asp, one His, and one Ser residue in the molecule. This enzyme prefers to hydrolyze peptide bonds on the carboxyl side of glutamic or aspartic acid.

Proteases, especially trypsin, are routinely used in cell culture to disperse single cells from tissues, and to passage adherent cells onto cell culture dishes (3,4). Trypsin (EC 3.4.21.4) is a serine protease from the PA clan (proteases of mixed nucleophile, superfamily A). Trypsin cleaves peptide chains mainly at the carboxyl sides of lysine or arginine. The enzymatic mechanism is similar to that of other serine proteases. These enzymes contain a catalytic triad consisting of histidine-57, aspartate-102, and serine-195 (5). These three residues form a charge relay that serves to make the active site serine nucleophilic. Trypsin-like proteases cleave peptide bonds following a positively charged amino acid (lysine or arginine). This specificity is driven by the residue which lies at the base of the enzyme's S1 pocket (generally a negatively charged aspartic acid or glutamic acid) (6).

Vesicular transport is a unique way of protein transport, widely existing in eukaryotic cells. Not only protein modification, processing and assembly are involved in protein transport, but also different directional vesicular transport and its complex regulation. Transferrin receptor (CD71) and glycophorin A (GPA) are highly-expressed erythroid cell surface markers. CD71 is a highly glycosylated transmembrane protein with multiple N-linked and O-linked oligosaccharide chains (7). GPA is also a highly glycosylated transmembrane protein with 1 N-linked oligosaccharide chain and 15 O-linked oligosaccharide chains (8). Therefore, the glycosylation process of CD71 and GPA may play a role in their expression and membrane localization.

Transferrin receptor plays a critical role in iron homeostasis by serving as a gatekeeper regulating iron uptake from transferrin. The transferrin receptor binds the serum iron-transport protein, transferrin, internalizes it through clathrin-coated pits, and facilitates transferrin-bound iron release in the sorting endosome. Iron-depleted transferrin returns to the cell surface where the protein is released for another cycle of iron transport (9). GPA is a major sialoglycoprotein of the human erythrocyte membrane required for malaria parasite invasion; it bears the antigenic determinants for the MNS blood groups. When human erythrocytes were treated with trypsin, GPA was removed from their surface, which implied erythrocyte cell surface localization of

this protein for attachment of the malaria parasite (10).

K562 is an erythroleukemia cell line derived from the pleural effusion of a patient with chronic myeloid leukemia in the acute transformation phase (11). K562 cells are widely used to study the mechanism of proliferation and differentiation of hematopoietic cells (12). Here we established a recovery model of CD71 and GPA distribution on the surface of K562 cells after protease digestion, and studied the effects of cytoskeletal and glycosylation inhibitors on their recovery. This model contributes to elucidating the molecular mechanisms of trans-membrane protein cycling and provides evidence on the role of the cytoskeleton in protein transport.

Materials and Methods

Cell culture

K562 cells were cultured in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA), 100 units/ml penicillin (Sigma-Aldrich, USA) and 100 μg/ml streptomycin (Sigma-Aldrich, USA) in a humidified incubator containing 5% CO_2 at 37 °C.

Digestion of K562 cells with proteases

Exponentially growing K562 cells were collected by centrifugation at 800 g for 5 min, and then washed three times with phosphate buffer saline (PBS). The cells were digested with S. griseus protease (Fluka, USA) or trypsin (Sigma-Aldrich, USA) (in PBS containing 0.02% EDTA) for 0−40 min at 37 °C, then 20% FBS was added to stop the digestion. The digested K562 cells were washed three times with PBS.

Cell viability analysis

Cell viability was assayed by trypan blue exclusion test. After K562 cells were digested with S. griseus protease (0, 0.5, 1.0, 1.5, 2.0 mg/ml) for 30 min at 37 °C, the cells were collected and stained with trypan blue; live and dead cells were counted using a haemocytometer.

Re-culture of protease-digested K562 cells

The digested K562 cells were washed three times with PBS and cultured in fresh RPMI 1640 medium supplemented with 10% FBS in a humidified incubator containing 5% CO_2 at 37 °C. After re-culture for different periods of time (0−24 h), the cells were collected by centrifugation to analyze cell surface expression of intact CD71 and GPA.

Treatment of protease-digested K562 cells with inhibitors

K562 cells were digested with S. griseus protease for 30 min, and then the un-digested and the digested K562 cells were exposed to 10 μg/ml colchicine (Sigma-Aldrich), 0.1 μg/ml paclitaxel (Sigma-Aldrich), 0.1 μg/ml cytochalasin D (Sigma-Aldrich), 0.1 μg/ml phalloidin (Invitrogen, USA), 25 μg/ml tunicamycin (Sigma-Aldrich) and 25 μg/ml benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) (Merck, USA), respectively. Cell surface expression of intact CD71 and GPA were analyzed by flow cytometry, after exposure to these chemicals for 3 h and 16 h respectively.

Flow cytometry analysis

Cell surface expression of intact CD71 and GPA were assayed by flow cytometry with a FACScan flow cytometer (Becton Dickinson). After the above-mentioned treatment, K562 cells were collected by centrifugation at 800 g for 5 min, rinsed twice with cold PBS containing 1% bovine serum albumin (BSA), and suspended in PBS. The suspended cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD71 antibodies (Caltag, USA) or FITC-conjugated anti-GPA antibodies (Caltag, USA) for 30 min at 4 °C. Mouse isotype IgG1-FITC (Caltag, USA) antibodies served as controls. After incubation, the cells were washed twice with cold PBS and analyzed immediately using FACScan instruments.

Statistical analysis

Experimental data were expressed as the mean ± standard deviation (SD). A two-tailed paired Student's t-test was used for analyses, which involved only two groups for comparison. And one-way or two-way ANOVA with Tukey's Honestly Significant Difference (HSD) test were used for analyses, which involved more than two groups for comparisons. Results at $P<0.05$ or less were considered statistically significant.

Results

Digestion of K562 cells with S. griseus protease decreased cell surface expression of intact CD71 and GPA

After K562 cells were digested with S. griseus protease for 30 min, the cell viability showed a concentration-dependent decrease (Table 1). And digestion with 1.0 mg/ml S. griseus protease for 30 min did not induce significant adverse effect on K562 cells.

After K562 cells were digested with 1.0 mg/ml S. griseus protease, cell surface expression of intact CD71 and GPA showed a time-dependent decrease (Figure 1). When the digestion last for 30 min, the expression of intact CD71 was less than 7% of the undigested level, and the expression of intact GPA was less than 1.5% of the undigested level. Therefore, digestion with 1.0 mg/ml S. griseus protease for 30 min resulted in an obvious decrease in cell surface expression of intact CD71 and GPA.

In addition, when the cells were digested with 1.0 mg/ml trypsin for 30 min, cell surface expression of intact CD71 and GPA showed a significant decrease, but the decrease was less compared with S. griseus pro-

Table 1. Effects of Protease at Different Concentrations on Viability of K562 Cells.

Protease concentrations (mg/ml)	Cell viability (%)
0	99.80±0.20
0.5	97.60±0.24
1.0	95.70±0.18
1.5	86.40±0.23*
2.0	82.60±0.36*

Values are presented as means ± SD (n=3). Statistical significance was determined by a one-way ANOVA with Tukey's HSD test and was defined as *$p<0.05$.

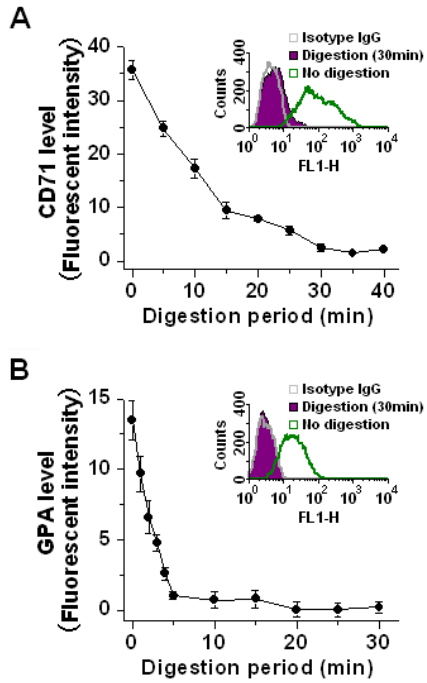

Figure 1. The expression of intact CD71 and GPA on the surface of K562 cells after protease digestion. After K562 cells were digested with 1.0 mg/ml S. griseus protease for indicated times, cell surface expression of intact CD71 (A) and GPA (B) were estimated by flow cytometry. Fluorescence distribution data displayed as single histograms for FL1-H represent cell surface expression of intact CD71 and GPA after protease digestion for 30 min. Error bars represent SD (n=3).

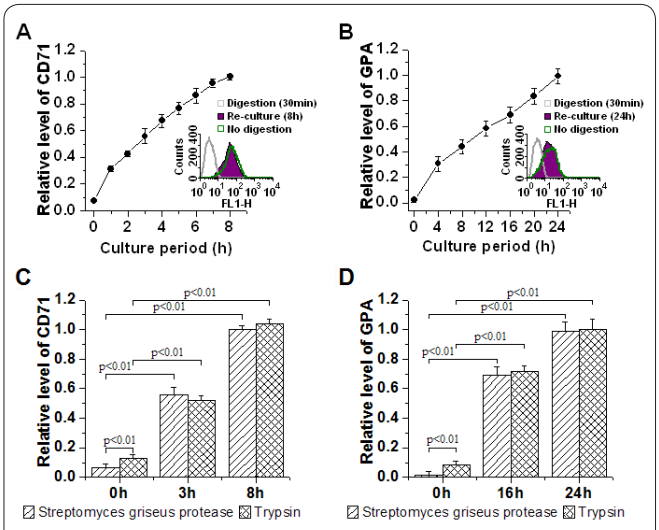

Figure 2. Recovery of intact CD 71 and GPA on cell surface of protease-digested K562 cells after re-culture for different periods of time. After K562 cells were digested with 1.0 mg/ml S. griseus protease or 1.0 mg/ml trypsin for 30 min, cell surface expression of intact CD71 (A, C) and GPA (B, D) were estimated by flow cytometry after re-culture for different periods of time. Fluorescence distribution data displayed as single histograms for FL1-H represent cell surface expression of intact CD71 and GPA after re-culture for 8 h and 24 h, respectively. Error bars represent SD (n=3). Statistical significance was determined by a two-way ANOVA with Tukey's HSD test.

tease-digested cells (Figure 2C and 2D).

Recovery of intact CD71 and GPA protein on the surface of protease-digested K562 cells after re-culture

Cell surface expression of intact CD71 and GPA showed a time-dependent increase after protease-digestion and re-culture. The expression of intact CD71 recovered to 56% of the undigested level after re-culture for 3 h, and it completely recovered to the undigested level after re-culture for 8 h (Figure 2A). The expression of intact GPA recovered to 69% of the undigested level after re-culture for 16 h, and it completely recovered to the undigested level after re-culture for 24 h (Figure 2B).

In addition, trypsin-digested cells showed similar cell surface recovery of intact CD71 and GPA to S. griseus protease-digested cells (Figure 2C and 2D).

Effects of microtubule depolymerizer and stabilizer on cell surface recovery of intact CD71 and GPA of protease-digested K562 cells

Vesicles trafficking membrane proteins or other substances are transported along the microtubules from one end to the other by motor proteins. Colchicine is a microtubule depolymerizing agent that inhibits microtubule polymerization by binding to tubulin. Paclitaxel on the other hand is a microtubule stabilizer, blocking dynamic instability by stabilizing GDP-bound tubulin in the microtubule. When undigested K562 cells were exposed to colchicine or paclitaxel, cell surface expression of intact CD71 and GPA showed no significant changes. However, when protease-digested K562 cells were re-cultured for 3 h, cell surface recovery of intact CD71 was significantly promoted by colchicine, while inhibited by paclitaxel (Figure 3A and 3C). When protease-digested K562 cells were re-cultured for 16 h, cell surface recovery of intact GPA was significantly inhibited by colchicine, while promoted by paclitaxel (Figure 3B and 3D).

Effects of microfilament depolymerizer and stabilizer on cell surface recovery of intact CD71 and GPA of protease-digested K562 cells

Vesicles can move along the actin microfilaments

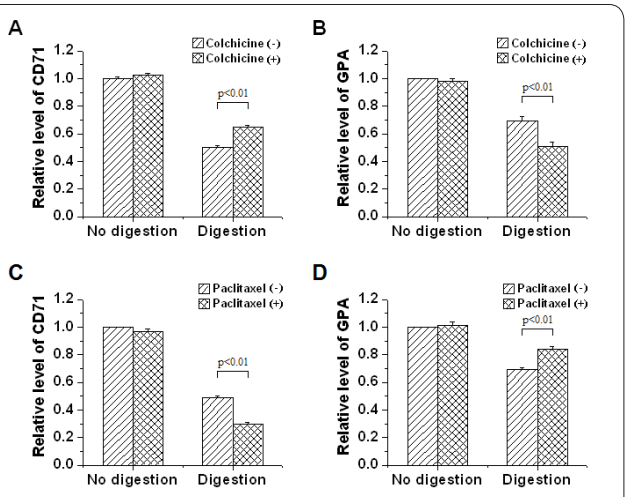

Figure 3. Effects of colchicine and paclitaxel on cell surface recovery of intact CD71 and GPA. After K562 cells were re-cultured with 10 μg/ml colchicine or 0.1 μg/ml paclitaxel, cell surface expression of intact CD71 and GPA were estimated by flow cytometry. Cell surface recovery of intact CD71 after re-culture with colchicine (A) or paclitaxel (C) for 3 h. Cell surface recovery of intact GPA after re-culture with colchicine (B) or paclitaxel (D) for 16 h. Error bars represent SD (n=3). Statistical significance was determined by a two-tailed paired Student's t-test.

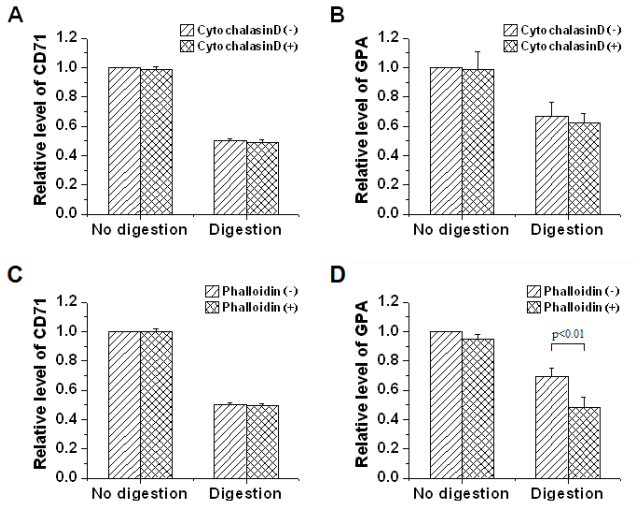

Figure 4. Effects of cytochalasin D and phalloidin on cell surface recovery of intact CD71 and GPA. After K562 cells were re-cultured with 0.1 µg/ml cytochalasin D or 0.1 µg/ml phalloidin, cell surface expression of intact CD71 and GPA were estimated by flow cytometry. Cell surface expression of intact CD71 after re-culture with cytochalasin D (A) or phalloidin (C) for 3 h. Cell surface expression of intact GPA after re-cultured with cytochalasin D (B) or phalloidin (D) for 16 h. Error bars represent SD (n=3). Statistical significance was determined by a two-tailed paired Student's t-test.

(F-actin) by myosin motor proteins. Cytochalasin D, a potent inhibitor of actin polymerization, is believed to bind to F-actin polymer and prevent polymerization of actin monomers. Conversely, phalloidin binds F-actin and prevents its depolymerization. When undigested K562 cells were exposed to cytochalasin D or phalloidin, cell surface expression of intact CD71 and GPA showed no significant changes. When protease-digested K562 cells were re-cultured for 3 h, cytochalasin D and phalloidin showed no effects on cell surface recovery of intact CD71 (Figure 4A and 4C). When protease-digested K562 cells were re-cultured for 16 h, phalloidin significantly inhibited cell surface recovery of intact GPA whereas cytochalasin D showed no effect on cell surface GPA recovery (Figure 4B and 4D).

Effects of glycosylation inhibitors on cell surface recovery of intact CD71 and GPA of protease-digested K562 cells

After CD71 and GPA are synthesized in the rough endoplasmic reticulum, they undergo glycosylation and become glycoproteins. Tunicamycin blocks N-linked glycosylation, and BADGP inhibits O-linked glycosylation. When undigested K562 cells were exposed to tunicamycin or BADGP, cell surface expression of intact CD71 and GPA showed no significant changes. When digested K562 cells were re-cultured for 3 h, tunicamycin significantly inhibited cell surface recovery of intact CD71, but BADGP showed no effect on cell surface CD71 recovery (Figure 5A and 5C). When protease-digested K562 cells were re-cultured for 16 h, tunicamycin and BADGP both significantly inhibited cell surface recovery of intact GPA (Figure 5B and 5D).

Discussion

Proteases are used to cleave the extracellular portion of transmembrane proteins of the erythrocyte (13,14). Rutledge indicated that removing 95% of the external domain of transferrin receptor has little effect on the turnover of the receptor (15). Enns reported the punctate fluorescence represented internalized ligand and receptor (16).

It has been reported that vesicular transport depends on the cell cytoskeleton. Membrane proteins stabilized their interactions with cytoskeletal microtubule proteins and microtubule associated proteins, and increased dynein to assist membrane protein transport (17). Cell membrane proteins are constantly in flux through endocytosis and exocytosis (18). Disruption of the cytoskeleton thus impedes cell transport and membrane protein localization on the cell surface (19).

Colchicine inhibits microtubule polymerization by binding to tubulin, which disrupts spindle formation during mitosis. Our study suggests that colchicine could block CD71 endocytosis but not its transport to the cell surface (20). Paclitaxel binds to 31 amino acid residues in the N-terminal amino acid and 217-231 amino acid residues of β3 tubulin (21,22). This increases the interactions among 13 protofilaments and enhances microtubule assembly, thereby stabilizing existing microtubules against depolymerization (23). Paclitaxel also inhibits the function of kinesins, a class of motor proteins that drive vesicular movement towards the plus end of microtubules (24). Our results suggest that this activity of paclitaxel probably blocked CD71 transport to the cell surface.

Cytochalasin D specifically binds to the positive end of F-actin, block subunit addition, break the balance between polymerization and depolymerization of microfilament, depolymerizate at the minus end of F-actin,

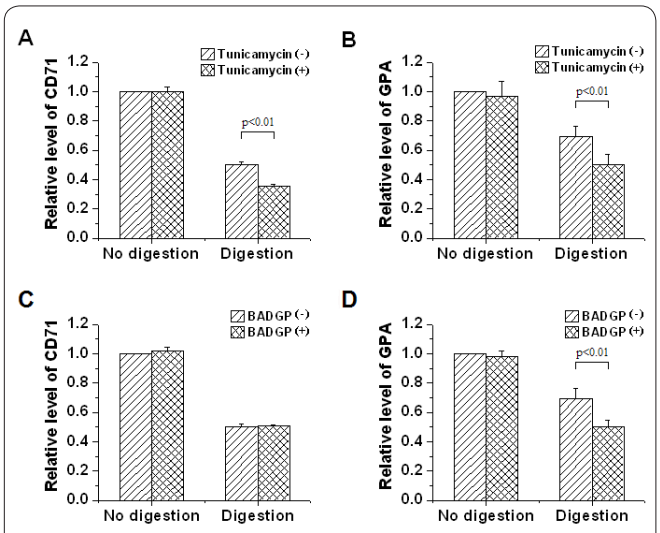

Figure 5. Effects of tunicamycin and BADGP on cell surface recovery of intact CD71 and GPA. After K562 cells were re-cultured with 25 µg/ml tunicamycin or 25 µg/ml BADGP, cell surface expression of intact CD71 and GPA were estimated by flow cytometry. Cell surface expression of intact CD71 after re-culture with tunicamycin (A) or BADGP (C) for 3 h. Cell surface expression of intact GPA after re-culture with tunicamycin (B) or BADGP (D) for 16 h. Error bars represent SD (n=3). Statistical significance was determined by a two-tailed paired Student's t-test.

selectively inhibit actin filament polymerization, and thus destroy the structure of microfilament. However this inhibitor had little effect on CD71 and GPA translocation and endocytosis. Phalloidin binds to aggregated microfilament, inhibits the disintegration of the microfilament, and breaks the dynamic balance between aggregation and disintegration. This may account for its ability to inhibit GPA transport to the cell surface, indicating that the stability of microfilaments may be important for GPA endocytosis.

Human TfR1/CD71 extracellular domain contains three N-linked glycosylation sites at Asn251, Asn317 and Asn727, and one O-linked glycosylation site at Thr104 (25,26,27,28). N-linked glycosylation is important for proper folding and transport of the protein to cell surface (29). Tunicamycin hinders the formation of the glycoprotein chain, forms new deglycosylated proteins, and inhibits the glycosylation of newly formed proteins in the endoplasmic reticulum, thus inhibiting N-linked protein glycosylation (30). Early reports suggested that tunicamycin had obvious inhibitory effects on cell surface expression of GPA (31), as well as the efficiency of CD71 localization on the cell surface (32). Our research found that treatment with tunicamycin on protease-digested K562 cells had a significant effect on the recovery of CD71 and GPA, suggesting that N-linked glycosylation had a role in CD71 and GPA membrane positioning. Elimination of the O-linked glycosylation enhances TfR1 cleavage and ectodomain release (33,34). Treatment with BADGP on protease-digested K562 cells had a significant effect on the recovery of GPA, indicating that inhibition of O-linked glycosylation prevents the formation of O-linked oligosaccharide chain on GPA, thus inhibiting its transport to the cell surface. Accordingly, O-linked glycosylation has a role in GPA membrane localization.

In conclusion, our research has established that using proteases, cytoskeletal and glycosylation inhibitors, it is possible to examine the mechanism of CD71 and GPA distribution or trafficking to the cell surface. This molecular mechanism may enable us better understand the dynamics of transmembrane protein transport and the important role of the cytoskeleton in that process.

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Interest Conflict

The authors declare that they have no conflict of interest.

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