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# Effect of Inhibitors of N-Linked Oligosaccharide Processing on the Cell Surface Expression of a Melanoma Integrin

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The role of trimming and processing of N-linked oligosaccharides on the cell surface expression of the melanoma vitronectin receptor, a member of the integrin family of cell adhesion receptors, was examined by using specific glucosidase and mannosidase inhibitors. Inhibition of glucosidases I and II by castanospermine or N-methyldeoxynojirimycin delayed the vitronectin receptor $\alpha/\beta$ chain heterodimer assembly and $\alpha$ chain cleavage and resulted in a decrease in the level of expression cell surface receptor. Conversely, the vitronectin receptor synthesized in the presence of the mannosidase I and II inhibitors, 1-deoxymannojirimycin and swainsonine, was transported normally to the cell surface with its $\alpha$ chain N-linked oligosaccharides in an endoglycosidase H–sensitive form. In the presence of swainsonine, time course studies of the cell surface replacement of control, endoglycosidase H–resistant receptor with an endoglycosidase H–sensitive form demonstrated a vitronectin receptor half-life of approximately 15–16 h. These studies provide evidence that the rates of assembly, proteolytic cleavage, and cell surface expression of the melanoma vitronectin receptor are dependent on the initial trimming of glucosyl residues from the $\alpha$ chain N-linked oligosaccharides.

**Key words: vitronectin receptor, adhesion receptor, castanospermine, N-methyldeoxynojirimycin, deoxymannojirimycin, swainsonine, intracellular transport**

The addition and maturation of N-linked oligosaccharide side chains constitute a common modification in the biosynthesis of both membrane-associated and secreted glycoproteins [1]. While the intracellular pathways of these two types of glycoproteins are believed to be distinct, the structure and modification processes of the N-linked oligosaccharides are similar for both classes of glycoproteins [2].

Following the cotranslational addition of core oligosaccharides to specific asparagine residues of a nascent glycoprotein, three glucosyl residues are removed from the $Glc_3Man_9GlcNAc_2$ structure by the glucosidase I and II enzymes. The trimming of

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mannose residues from this "high mannose" form by the mannosidase I and II enzymes and the addition of GlcNAc, Gal, and sialic acid residues result in the formation of a "complex" form of N-linked oligosaccharides [1]. While much is known about the structure and synthesis of N-linked oligosaccharides, the role that they play in the intracellular transport and function of glycoproteins is not clearly understood.

Inhibitors that block the activities of the specific enzymes involved in the maturation of N-linked oligosaccharides have been valuable in assessing their role in a variety of systems [3,4]. For example, the biosynthesis and function of the insulin and insulin-like growth factor receptors have been dissected by using the glucosidase I and II inhibitors castanospermine (CSP) and N-methyldeoxynojirimycin (NM-DNJ) and the mannosidase I and II inhibitors 1-deoxymannojirimycin (DMJ) and swainsonine (SSN). These studies demonstrated that inhibition of N-linked oligosaccharide glucose trimming, but not the subsequent processing steps, delayed the proteolytic processing, cell surface expression, and function of the insulin receptor [5–7].

In the present study, we have analyzed the role of N-linked oligosaccharide processing on the cell surface expression of a human melanoma vitronectin receptor. This receptor is a member of the integrin family of cell adhesion receptors that specifically interact with Arg-Gly-Asp (RGD) amino acid sequences of extracellular matrix proteins [8,9]. Like other members of this family, the melanoma vitronectin receptor is a heterodimer composed of an $\alpha$ chain subunit that is proteolytically cleaved into disulfide-bonded heavy and light chain components and a non-covalently associated $\beta$ chain. Both the $\alpha$ and $\beta$ chains contain N-linked oligosaccharides and the majority of those on the $\alpha$ chain are processed to the complex form [10]. To examine the role of N-linked oligosaccharide processing in the biosynthesis of the melanoma vitronectin receptor, we have tested the effects of specific N-linked oligosaccharide-processing inhibitors on its cell surface expression.

## MATERIALS AND METHODS

### Materials

SSN was obtained from Boehringer Mannheim (Indianapolis, IN); NM-DNJ, DMJ, CSP, and endoglycosidase H (Endo H, Endo-$\beta$-N-acetylglucosaminidase H, EC 3.2.1.96) were obtained from Genzyme (Boston, MA). Protein A–Sepharose was purchased from Sigma Chemical Co. (St. Louis, MO).

### Cell Lines and Antibodies

The UCLA-M21 cell line (M21) is a human melanoma cell line obtained from Dr. D.L. Morton (University of California at Los Angeles). M21 cells were maintained in either suspension or adherent cultures in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (FCS). A375-P melanoma cells were obtained from Dr. I.J. Fidler, M.D. Anderson Hospital and Tumor Institute, Houston, TX. Monoclonal antibody (mAb) 142 [11] that reacts with the $\alpha$ chain of the melanoma vitronectin receptor was obtained from D.A. Cheresh, Research Institute of Scripps Clinic, La Jolla, CA.

### Treatment With Inhibitors

Glucosidase and mannosidase inhibitors were used at optimum concentrations determined previously for M21 and A375-P melanoma cells (256 $\mu$g/ml CSP, 2 mM

NM-DNJ, 2 mM DMJ, 4 μg/ml SSN) [12]. Stock solutions of inhibitors were made and serially diluted in the growth media described above. The cells were preincubated in the presence of the inhibitors for 1 to 48 h prior to radiolabeling, depending on the nature of the experiment as described in the text and figure legends. No significant loss of cell viability resulted from inhibitor treatments as judged by trypan blue exclusion and/or counts per minute of [$^{35}$S]methionine incorporated into protein. Standardization of samples was achieved through the use of equivalent numbers of cells throughout all experiments.

### Pulse-Chase Biosynthetic Labeling

Biosynthetic labeling studies were performed as previously described [10,11]. Briefly, M21 cells were harvested and preincubated for 15 min in L-methionine-free RPMI 1640 (Selectamine, GIBCO) containing 10% dialyzed FCS and plus or minus inhibitors prior to a 10 min biosynthetic pulse with 1 mCi/ml [$^{35}$S]methionine (1,295 Ci/mmol, Amersham). After the pulse, cells were washed three times by centrifugation in Hank's balanced salt solution containing 4 mM L-methionine, and the cell aliquot representing the zero time point was removed, washed, and lysed as described below. The remaining cells were then resuspended in warmed, complete RPMI 1640 plus and minus inhibitors and were recultured at 37°C. At time intervals designated in the figure legends, cell aliquots ($5 \times 10^6$ cells per time point) were removed, immediately solubilized, and prepared for immunoprecipitations as described below.

### Cell Surface Iodination

Cell surface forms of the vitronectin receptor were analyzed by labeling surface molecules with [$^{125}$I] by using Enzymobeads (BioRad) according to the manufacturer's protocol. Cell cultures were harvested after treatment with inhibitors for various times described in the text and washed three times with cold PBS; then each cell pellet ($10^7$ cells) was resuspended in 50 μl of 0.2 M sodium phosphate buffer, pH 7.2. Following the addition of 50 μl of rehydrated Enzymobeads, cells were labeled with 1 mCi [$^{125}$I] (Amersham, 583.7 MBq, 15.8 mCi/μg). Cell lysates were then prepared as described below.

### Indirect Immunoprecipitation and Gel Electrophoresis

Immunoadsorbants of purified mAb 142 ($IgG_1$) were prepared by covalent conjugation to agarose beads (Affi-gel 10, Bio-Rad Laboratories, Richmond, CA) at 2 mg/ml of beads. Beads were prepared as a 10% (v/v) suspension and 100 μl was washed twice in PBS/0.5% Tween 20 (v/v) and used for immunoprecipitation. Immunoprecipitations were done with an excess of antibody to allow the quantitative assessment of the results. Iodinated cells were prepared for analysis by lysis in RIPA buffer (0.1 M Tris-HCl, pH 7.2, 0.15 M NaCl, 1% (w/v) deoxycholate, 1% (w/v) Nonidet P-40, 0.1% (w/v) SDS, 1% aprotinin, and 2 mM phenylmethylsulfonyl fluoride) [10,11]. Prior to immunoprecipitation, lysates were cleared by ultracentrifugation at 40,000 rpm in a Beckman SW 50.1 rotor for 45 min and stored at −70°C until use. Immunoprecipitation was performed by incubating cell lysates with antibody beads overnight at 4°C. The antigen containing beads were then washed five times with PBS/0.5% Tween 20 and resuspended in 90 μl of Laemmli sample buffer [13] and boiled for 5 min. Sensitivity of N-linked oligosaccharides to digestion with Endo H was analyzed by suspension of antigen-containing beads

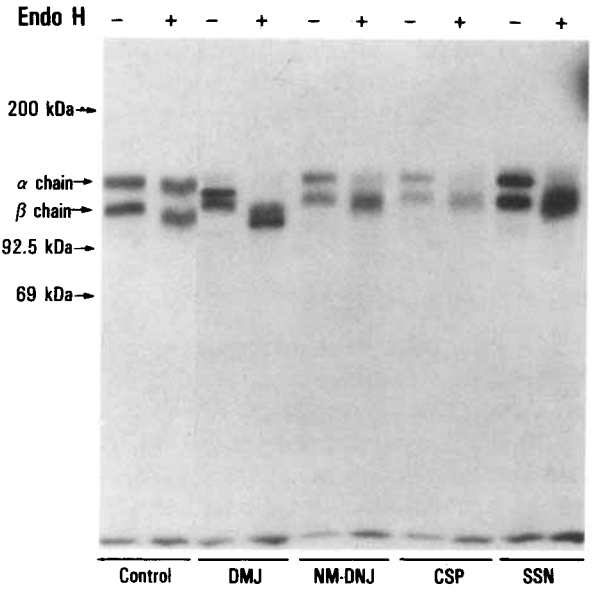


Fig. 1. Effect of glucosidase and mannosidase inhibitors on the cell surface expression of the melanoma vitronectin receptor. Human A375-P melanoma cells were incubated in the presence of glucosidase or mannosidase inhibitors (256 μg/ml CSP, 2 mM NM-DNJ, 2 mM DMJ, 2 μg/ml SSN) for 24 h prior to $^{125}$I-cell surface labeling. Labeled vitronectin receptor was then immunoprecipitated with mAb 142, digested with Endo H (+ lanes), and analyzed on a 7.5% SDS-PAGE gel under reducing conditions.

in 10 μl of 20 mM sodium citrate buffer, pH 5.5, plus and minus 0.01 units of Endo H. After a 2 h incubation at 37°C, 10 μl of 2× sample buffer was added and each sample was boiled for 5 min. The presence or absence of β-mercaptoethanol is indicated in the text and figure legends. Immunoprecipitated proteins were analyzed by SDS-PAGE and visualized according to previously described methods [10,11]. Quantitative analysis of SDS-PAGE gels was performed by laser densitometry scanning of the autoradiographs.

## RESULTS AND DISCUSSION

### Effect of Glucosidase and Mannosidase Inhibitors on the Cell Surface Expression of the Melanoma Vitronectin Receptor

The requirement of N-linked oligosaccharide trimming and processing for the cell surface expression of the vitronectin receptor was examined by $^{125}$I-surface labeling cells that had been incubated in the presence of the glucosidase and mannosidase inhibitors for 24 h. Aliquots of equivalent numbers of A375-P human melanoma cells were treated for 24 h with either the glucosidase inhibitors NM-DNJ and CSP or the mannosidase inhibitors DMJ and SSN at the indicated concentrations. Cell surface molecules were then $^{125}$I-labeled and the vitronectin receptor was immunoprecipitated with mAb 142 and resolved by SDS-PAGE (Fig. 1).

As described previously, the vitronectin receptor isolated from human melanoma cells migrates by SDS-PAGE under reducing conditions as a heterodimer consisting of a 130 kDa, Endo H–resistant α chain and a 105 kDa, Endo H–sensitive β chain [10,11] (Fig. 1). The 27 kDa light chain that results from the posttranslational proteolytic

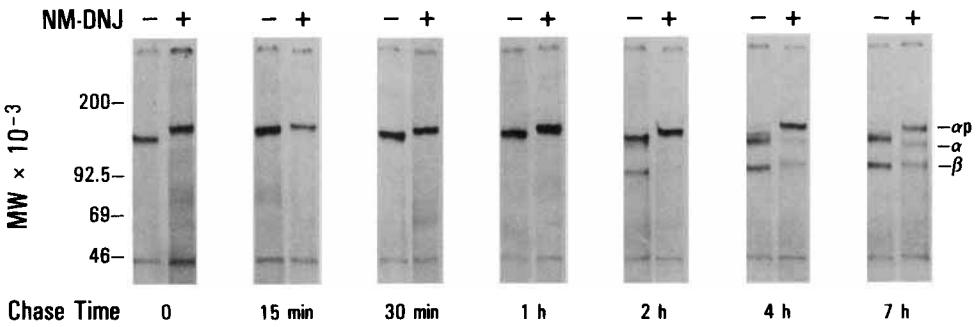


Fig. 2. Biosynthesis of the melanoma vitronectin receptor in the presence and absence of NM-DNJ. Human M21 melanoma cells were pulse-labeled in [$^{35}$S]methionine for 10 min and then chased in unlabeled media for the indicated time intervals. Duplicate samples were done in the presence and absence of 2 mM NM-DNJ throughout the labeling and chase periods. The melanoma vitronectin receptor was then immunoprecipitated mAb 142 and analyzed on a 4–12% gradient SDS-PAGE gel under reducing conditions. The uncleaved precursor $\alpha$ chain form is designated as $\alpha_p$.

cleavage of an $\alpha$ chain precursor is not visible when the samples are analyzed on a 7.5% SDS-PAGE gel.

Treatment of the cells with the glucosidase inhibitors CSP and NM-DNJ reduces the level of vitronectin receptor expressed at the cell surface by approximately 80% and 50%, respectively, when compared to control cells (Fig. 1). Pulse-chase [$^{35}$S]methionine biosynthetic labeling experiments with NM-DNJ-treated cells demonstrates no effect on the level of $\alpha$ chain synthesis but a delay in the maturation of the vitronectin receptor (Fig. 2). A mature receptor (cleaved $\alpha$ chain and associated $\beta$ chain) begins to appear in control cells between 1 and 2 h into the chase period and the conversion is complete by the 7 h time point (Fig. 2) [10]. Mature receptor does not appear in NM-DNJ-treated cells until 4 h of the chase and a precursor, uncleaved $\alpha$ chain form ($\alpha_p$) is still present after 7 h of the chase. Note that the NM-DNJ $\alpha_p$ form migrates somewhat slower than the control form. The presence of glucosyl residues on the N-linked oligosaccharides ($Glc_3Man_9GlcNAc_2$ forms) of the vitronectin receptor must, therefore, retard the electrophoretic migration of the $\alpha_p$ form, delay its intracellular transport, and possibly cause premature receptor degradation. The result is a decrease in the level of vitronectin receptor expressed at the cell surface. The observed Endo H sensitivity of the $\alpha$ chain expressed at the cell surface in CSP- and NM-DNJ-treated cells is consistent with the presence of glucosylated N-linked oligosaccharides [3,4]. The migration of the cell-surface-expressed $\alpha$ chain in NM-DNJ or CSP-treated cells more closely resembles the control form (Fig. 1). This is most likely due to the removal of one or two mannose residues by mannosidase I that can occur on fully glucosylated N-linked oligosaccharides as reported for other glycoproteins synthesized in the presence of CSP and NM-DNJ [14,15].

The importance of the removal of glucosyl residues from the N-linked oligosaccharides of the vitronectin receptor is further supported by the observation that no inhibition of receptor cell surface expression is observed in cells treated with the mannosidase inhibitors DMJ and SSN (Fig. 1). In the presence of SSN there is a two-fold increase in the levels of receptor expressed at the cell surface. As with CSP and NM-DNJ, the Endo H sensitivity of the $\alpha$ chain expressed at the cell surface under the influence of DMJ or

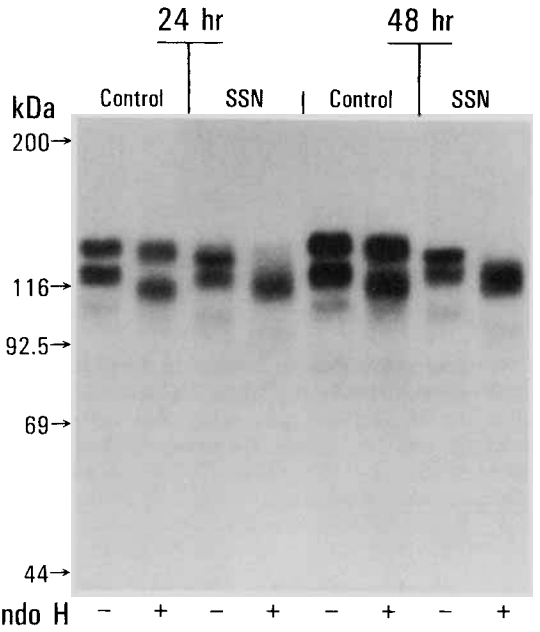


Fig. 3. Cell surface expression of the melanoma vitronectin receptor in the presence of SSN. Human M21 melanoma cells were cultured in the presence and absence of 2 $\mu$g/ml SSN for 24 and 48 h prior to $^{125}$I-cell surface labeling. The melanoma vitronectin receptor was then immunoprecipitated with mAb 142, digested with Endo H (+ lanes), and analyzed on a 7.5% SDS-PAGE gel under reducing conditions.

SSN confirms the effect of these inhibitors. The $\alpha$ chain expressed in the presence of DMJ also exhibits a faster migration compared to the control form due, most likely, to the presence of "high mannose" $Man_{5-9}GlcNAc_2$ N-linked oligosaccharides that result from the inhibition of mannosidase I [3,4]. Endo H digestion of this form results in an $\alpha$ chain that migrates as a deglycosylated $\alpha$ chain minus the 27 kDa light chain (under reducing conditions) (Fig. 1). The $\alpha$ chain expressed in the presence of SSN migrates slightly faster than the control form prior to Endo H digestion, consistent with the presence of "hybrid" N-linked oligosaccharides (half high mannose and half complex) (Figs. 1, 3) [16]. Endo H digestion results in an $\alpha$ chain form that comigrates with the $\beta$ chain as a diffuse band that is less Endo H sensitive than the form expressed in DMJ-treated cells (Figs. 1, 3).

After 24 h of treatment with SSN, approximately 80% of the cell surface vitronectin receptor has been replaced with a form containing an Endo H–sensitive $\alpha$ chain, and the replacement is complete by 48 h (Fig. 3). Based on the reported actions of the glucosidase and mannosidase inhibitors, the vitronectin receptor synthesized in the presence of DMJ vs. NM-DNJ or CSP should differ only by the presence of one to three glucosyl residues on its N-linked oligosaccharides [3,4]. Thus, although complete processing of the N-linked sugars from the high mannose to the complex form is not required for cell surface expression of the vitronectin receptor, the failure to remove the three glucosyl residues from the N-linked oligosaccharides appears to substantially delay its maturation and surface expression.

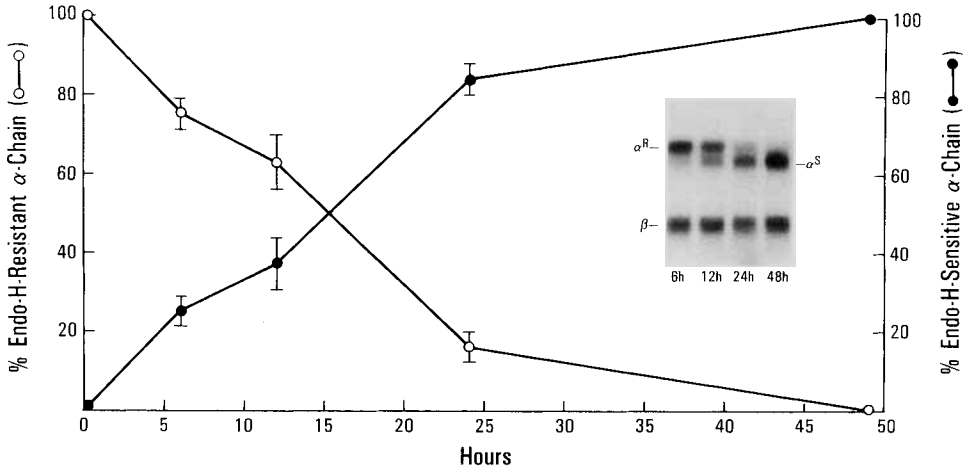


Fig. 4. Kinetics of the turnover of the melanoma vitronectin receptor in the presence of SSN. Aliquots of human M21 melanoma cells were incubated in the presence of 2 μg/ml SSN for 6, 12, 24, and 48 h prior to $^{125}$I-cell surface labeling. The melanoma vitronectin receptor was then immunoprecipitated with mAb 142, digested with Endo H, and analyzed on a 7.5% SDS-PAGE gel under non-reducing conditions (**inset**). The percentage of Endo H–resistant and –sensitive α chain was calculated by densitometric scanning of the autoradiograph. Points represent the mean of duplicate samples with the range of values indicated.

The presence of glucosylated N-linked oligosaccharides has no apparent effect on α/β chain association or α chain cleavage. Therefore, these posttranslational modifications are not dependent on the processing of N-linked oligosaccharides once the glucosyl residues have been removed. Moreover, no effect on migration of the β chain subunit is observed in the presence of the glucosidase and mannosidase inhibitors used here (Figs. 1, 3, and Fig. 4, inset). This supports the hypothesis that the N-linked oligosaccharides of the β chain may be inaccessible to the processing enzymes due, most likely, to a conformational constraint.

The findings presented here are analagous to those previously reported for the effects of glucosidase and mannosidase inhibitors on the expression of several other cell surface proteins including the insulin and insulin-like growth factor receptor, the melanoma-associated proteoglycan, and the epidermal growth factor receptor [5–7,12,17]. Possible mechanisms that have been proposed for the delay in maturation due to glucosylated N-linked oligosaccharides include effects on protein conformation or oligomerization reactions that may be required for proper transfer from the rough endoplasmic reticulum (rER). In addition, it has been suggested that the glucosyl residues may also be acting as retention signals in the rER [5,18–22].

## Kinetics of the Turnover of the Melanoma Vitronectin Receptor

A more detailed kinetic analysis of the replacement of the cell surface vitronectin receptor with the SSN-treated form was performed in order to approximate the turnover time of this receptor in melanoma cells. M21 cells were iodinated after 6, 12, 24, and 48 h of culture in SSN. The vitronectin receptor was immunoprecipitated from the iodinated cell lysates with Mab 142, digested with Endo H, and analyzed by SDS-PAGE under non-reducing conditions for better resolution of the α and β chains (Fig. 4, inset). The

percentage of Endo H–resistant and –sensitive $\alpha$ chain was calculated by densitometric scanning of the autoradiograph. The rate of replacement of the cell surface vitronectin receptor was then calculated by plotting the appearance and disappearance of Endo H-sensitive and –resistant $\alpha$ chain forms, respectively, vs. the time cultured in the presence of SSN (Fig. 4). These data demonstrate a half-life for the vitronectin receptor of approximately 15–16 h. We do not know whether the turnover of this receptor involves its internalization and subsequent recycling or degradation or whether it is released from the cell surface. Moreover, we cannot rule out the possibility that the turnover rate in the presence of SSN differs from that of untreated cells, particularly since SSN treatment resulted in an apparent increase in the level of cell surface receptor (Fig. 1). If the cell surface turnover of receptor involves internalization and degradation, the inhibition of lysosomal mannosidase activity by SSN could affect the turnover rate [3,4]. This may also account for the two-fold increase in cell surface expression of the vitronectin receptor observed in the presence of SSN (Figs. 1, 3). Nonetheless, replacement of cell surface antigens in the presence of mannosidase I and II inhibitors such as SSN and DMJ provides an effective method for antigen half-life approximations.

In summary, we described here some of the requirements for the assembly and cell surface expression of the melanoma vitronectin receptor, a member of the integrin family of adhesion receptors. The results presented in this report demonstrate that the presence of glucosyl residues on the N-linked oligosaccharides of the $\alpha$ chain subunit delays $\alpha/\beta$ chain association, $\alpha$ chain cleavage, and efficient transport of the mature receptor to the cell surface. In addition, we have demonstrated an approximate receptor half-life of 15–16 h by measuring the replacement of cell surface receptor in the presence SSN. These preliminary results make it possible to investigate the mechanisms of turnover of the vitronectin receptor and to address whether changes in turnover rate are induced by interactions with various types of adhesive substrates.

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