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Insulin-Induced Translocation of Glucose Transporters to the Plasma Membrane Precedes Full Stimulation of Hexose Transport†

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ABSTRACT: Insulin stimulation of hexose transport in 3T3-L1 adipocytes was studied at 27 °C. At this temperature, the transport of 2-deoxyglucose was stimulated 8-fold, with a half-time of 9.5 min. Under the same conditions, the increase in cell surface glucose transporters, as measured by labeling in the intact cell with galactose oxidase and tritiated borohydride, was only 2.6-fold. Moreover, the half-times for the increase in cell surface glucose transporters and for the decrease in transporter number in the intracellular pool were both 4 min. Thus, these processes clearly precede the full stimulation of transport. These data are in agreement with immunolocalization studies of the glucose transporter in this cell line and further support the hypothesis that a second mechanism besides translocation is involved in the stimulation of hexose transport by insulin [Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W., & Gueze, H. J. (1988) *J. Cell Biol. 106*, 69–76]. The findings presented here indicate that neither the translocation of glucose transporters to, nor their subsequent insertion into, the plasma membrane is the rate-limiting step in the stimulation of hexose transport by insulin. Rather, there is a second mechanism of activation, which is rate limiting and occurs after the transporter is in the plasma membrane.

Insulin treatment of fat and muscle cells leads to the translocation of glucose transporters from an intracellular site to the plasma membrane; this process results in an increase in the rate of glucose transport (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Kono et al., 1981, 1982; Karnielli et al., 1981). However, several recent studies have suggested that a second mechanism besides translocation may contribute to the stimulation of glucose transport by insulin. In 3T3-L1 adipocytes, the increase in the glucose transporter content of the plasma membrane upon insulin challenge was determined

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by immunoelectron microscopy and found to be 3-fold, as compared to a 10–20-fold stimulation of transport rate under the same conditions (Blok et al., 1988). Furthermore, our laboratory has recently developed a procedure to quantitate the cell surface glucose transporters in intact 3T3-L1 adipocytes; by use of this technique, it was found that the concentration of cell surface glucose transporters increases 2.6-fold, while the transport rate is increased by 11-fold (Calderhead & Lienhard, 1988). In addition to results in this cell type, several recent reports have provided suggestive evidence for the occurrence of a second mechanism for stimulation of transport by insulin in rat adipocytes. Baly and Horuk (1987) found that the rate of hexose transport into plasma membrane vesicles isolated from cycloheximide- and insulin-treated rat adipocytes is 12-fold greater than into vesicles isolated from basal cells, even though there is only a 1.6-fold increase in the glucose transporter content, as determined by cytochalasin B binding. In addition, Joost et al. (1988) have shown that the 40-fold increase in the rate of hexose transport in rat adipocytes induced by insulin is accompanied by a considerably smaller increase in the glucose transporter content of the isolated plasma membranes (either 7-fold, as assessed by cytochalasin B binding, or 2-fold, as determined by immunoblotting).

In this study, we have used a kinetic approach to examine further the characteristics of this second mechanism of activation in 3T3-L1 adipocytes. The time courses at 27 °C for the stimulation of hexose transport, for the translocation of glucose transporters from their intracellular location, and for the increase in the cell surface glucose transporter content in response to insulin were determined. The results from this study provide further evidence for the existence of a second mechanism involved in the stimulation of glucose transport by insulin. We show that the insulin-stimulated increase in cell surface glucose transporters clearly precedes the full stimulation of transport; thus, this second mechanism is the rate-limiting step in the stimulation of hexose transport by insulin.

Materials and Methods

Materials. 2-Deoxy[1,2-^{3}H]-D-glucose was from ICN, sodium [^{3}H]borohydride (20 Ci/mmol) from Amersham, and [*methyl*-^{14}C]-3-*O*-methyl-D-glucose from New England Nuclear. Phloretin was from Gallard Schlessinger Corp. Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) from *Dactylium dendroides* was purchased from Sigma and suspended in 150 mM NaCl–10 mM sodium phosphate, pH 7.4 (PBS[1]); inherent protease activity was inactivated by incubating the solution at 50 °C for 30 min in a water bath, and aliquots were stored at −70 °C prior to use (Gahmberg, 1978). Octaethyleneglycol dodecyl ether was from Nikkol Chemical Co. All other reagents were as previously described (Gibbs et al., 1986; Biber & Lienhard, 1986).

Cell Culture. Monolayers of 3T3-L1 fibroblasts were grown in 35-mm culture plates and differentiated into adipocytes as previously described (Frost & Lane, 1985). Cells were incubated in serum-free Dulbecco's modified Eagle's medium (DMEM) for 2 h at 37 °C prior to use.

Hexose Transport Assays. Plates were washed three times with 3 mL of Krebs–Ringer phosphate buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, and 5 mM sodium phosphate, pH 7.4) (KRP) at 27 °C and then covered with 1 mL of KRP. All plates were incubated in KRP at 27 °C for 30 min (except for the plates exposed to insulin for 1 h), with 1 μM insulin present for the times shown. After the 30 min, deoxyglucose uptake was measured by the addition of [^{3}H]deoxyglucose (final concentration, 50 μM or 5 μM, and 0.5 μCi/mL) for 1 min, immediately followed by three washes with 3 mL of ice-cold PBS. The cells were solubilized in 1 mL of 1% Triton X-100, and the tritium in an aliquot was measured by liquid scintillation spectrometry. The values for uptake were corrected for the nonspecific association of [^{3}H]deoxyglucose with the cells by subtracting the uptake in the presence of 25 μM cytochalasin B, a potent inhibitor of transport (Bloch, 1973). The values for the nonspecifically associated [^{3}H]deoxyglucose were the same for basal and fully stimulated cells and amounted to ~40% of the uptake by basal cells and ~5% of the uptake in maximally stimulated cells. When used, cytochalasin B was present throughout the 30-min preincubation period.

The transport of 3-*O*-methylglucose was measured by incubating cells with the hexose (50 μM and 0.25 μCi/mL) for 30 s after various periods of exposure to insulin. The plate was then rapidly washed three times with 100 μM phloretin in PBS at 4 °C. The cells were then solubilized, and the radioactivity was measured as described above. Values were corrected for extracellular isotope by subtraction of the values for cell-associated radioactivity in the presence of 25 μM cytochalasin B. Because fully stimulated cells equilibrated with 3-*O*-methylglucose in ~2 min [Frost and Lane (1985); data not shown], it was necessary to convert the observed rate of transport to obtain a true initial rate. This correction was carried out as described previously for rat adipocytes (Foley et al., 1978).

Determination of Cell Surface Glucose Transporter Levels. Glycoproteins at the cell surface, including the glucose transporter, were labeled by treating intact cells with galactose oxidase and then subsequent reduction with [^{3}H]borohydride (Gahmberg, 1978). The glucose transporter was then isolated by immunoprecipitation. The details of this method and the validation of it as a measure of the relative amount of surface transporter are reported elsewhere (Calderhead & Lienhard, 1988). A brief description follows. Plates were washed three times with 3 mL of KRP at 27 °C and then covered with 1 mL of KRP and treated with insulin for the required time, exactly as described for hexose transport. After the 30-min incubation, the monolayers were washed three times with 3 mL of an ice-cold phosphate buffer (117 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 20 mM sodium phosphate, pH 7.4); subsequent procedures were performed at 4 °C with 1 mL of this buffer per plate. Cell surface galactose residues were oxidized by 1-h incubation with galactose oxidase (40 units per plate; units as defined by Sigma) and then reduced with carrier-free [^{3}H]$NaBH_4$ (2 mCi per plate) for 10 min. After being washed with buffer, the cells were solubilized in 1.5 mL of 2% octaethyleneglycol dodecyl ether in PBS containing protease inhibitors (4 mM EDTA, 5 mM *N*-ethylmaleimide, 1 μg/mL pepstatin A, 10 μM L-*trans*-epoxysuccinylleucylamido-3-methylbutane, and 200 μM diisopropyl fluorophosphate). The lysate was centrifuged at 180000g_{max} for 1 h. The glucose transporter was immunoprecipitated from the supernatant as described previously, using affinity-purified antibodies against the carboxy-terminal peptide of the transporter (Gibbs et al., 1986; Davies et al., 1987). These antibodies were isolated from antiserum provided by Dr. Stephen A. Baldwin. SDS samples of the immunoprecipitates

[1] Abbreviations: PBS, phosphate-buffered saline; KRP, Krebs–Ringer phosphate; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's-modified Eagle's medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

(equivalent to 10^6 cells) were electrophoresed on 10% polyacrylamide gels; the gels were sliced and the slices incubated in 6% Protosol in Econofluor (National Diagnostics) at 37 °C with agitation overnight. The radioactivity in the slices was measured by liquid scintillation spectrometry. For each sample the total radioactivity in the transporter peak was determined.

A number of control experiments were performed. Ezaki & Kono (1982) have found that incubation of rat adipocytes at 10 °C for 4 h leads to translocation of the glucose transporter to the plasma membrane to the extent of ~25% of the translocation elicited by insulin at 37 °C. In order to determine the effect of incubation at 4 °C during the labeling procedure on the level of cell surface glucose transporters in 3T3-L1 adipocytes, cells were labeled with galactose oxidase and [^{3}H]$NaBH_4$ immediately upon chilling (as described above) or after 1-h incubation at 4 °C. No difference in the amount of label in the glucose transporter was found. This result indicates that the incubation at 4 °C for 1 h does not in itself result in significant translocation of the glucose transporters (data not shown). In order to determine whether insulin could produce translocation at 4 °C, 1 μM insulin was added to some plates for the 1-h incubation with galactose oxidase and to others for 1 h at 4 °C prior to subsequent galactose oxidase treatment; in both cases, no increase in cell surface glucose transporters was observed compared to control (no insulin) cells (data not shown). These experiments indicate that the 4 °C wash used to stop translocation at different times following insulin challenge was effective. Finally, in the absence of galactose oxidase there was no detectable incorporation of radiolabel into the transporter (D. M. Calderhead, personal communication).

Determination of Intracellular Glucose Transporters. In order to measure insulin-induced changes in the glucose transporter content of the intracellular membranes, a simple fractionation procedure was developed. The plasma membrane of 3T3-L1 adipocytes was labeled with galactose oxidase and [^{3}H]$NaBH_4$. After labeling and homogenization, the distribution of galactose oxidase specific label was followed upon centrifugation. It was found that centrifugation at $16000g_{max}$ for 20 min pelleted 95% of the galactose oxidase specific label and that the remaining supernatant contained 60% of the total cell glucose transporter. Following insulin stimulation at 37 °C, the transporter content of the supernatant decreased by half. Thus, this supernatant contains a substantial portion of the insulin-responsive, intracellular glucose transporters (Gould et al., submitted for publication).

Cells were treated as described above for determination of the rate of stimulation of transport. Following exposure to insulin for the stated time, the monolayers were immediately washed twice with 3 mL of ice-cold 270 mM sucrose, 20 mM Hepes, and 1 mM EDTA, pH 7.4, scraped from the plate into 6 mL of this buffer containing the protease inhibitors 1 μg/mL pepstatin A, 10 μM L-*trans*-epoxysuccinylleucylamido-3-methylbutane, 200 μM diisopropyl fluorophosphate, and 0.2 mM phenylmethanesulfonyl fluoride, and homogenized by 25 hand-driven strokes of a 30-mL glass homogenizer with Teflon pestle (Thomas 3431-D88, clearance 0.13–0.18 mm). The homogenate was centrifuged at $16000g_{max}$ for 20 min at 4 °C in a 12-mL tube in a Sorval SS34 rotor. The relative transporter content of this supernatant was determined by quantitative immunoblotting as described previously (Biber & Lienhard, 1986), with the following modifications. Samples for electrophoresis were dissolved in SDS sample buffer containing 4% SDS (Pierce, lauryl grade), 25 mM dithiothreitol, 1 mM EDTA, 0.002% bromophenol blue, 100 mM Tris-HCl, pH 6.8, and the same protease inhibitors as were present in the homogenization buffer. After electrophoresis, proteins were transferred to nitrocellulose (0.2 μm, Schliecher & Scheull type BA83) by using a Hoefer TE22 electroblotter at 250 mA for 45 min in 25 mM sodium phosphate buffer, pH 6.5.

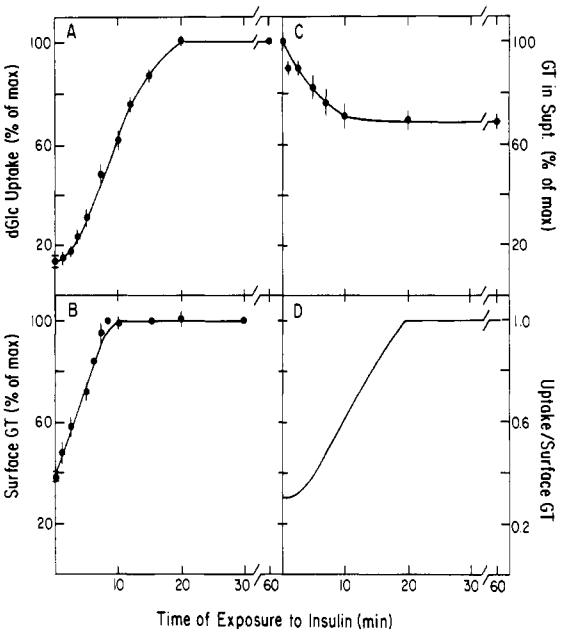

FIGURE 1: Time courses for insulin action. Panel A shows the time course of insulin stimulation of 2-deoxyglucose uptake at 27 °C. Four separate experiments were performed, and the rates at each time point were expressed as a percent of the rate measured after exposure to 1 μM insulin for 60 min. The figure presents the mean ± SEM of the four experiments. The time of exposure to insulin does not include the 1-min assay time (see Materials and Methods). The actual values of 2-deoxyglucose uptake for basal and fully stimulated adipocytes were 29.5 ± 2.6 and 242 ± 1.5 pmol/10^6 cells per min. Panel B shows the time course for the insulin-stimulated increase in glucose transporters at the cell surface, determined by quantitation of the radioactivity in the region of the gel corresponding to the glucose transporter (see Materials and Methods). The amount of surface transporter is presented as the ratio of the radiolabel in this peak after various periods of exposure to 1 μM insulin to that in the peak after 30-min exposure. The data are the mean and SEM from four separate determinations of the time course. The points without error bars are those from a few additional time points, only performed in one of the experiments. The value for the stimulation in the amount of surface transporter after 30 min of exposure to 1 μM insulin was (2.6 ± 0.09)-fold (SEM, $n = 4$). Panel C shows the time course of insulin-stimulated translocation of the glucose transporters from the intracellular membranes at 27 °C. The relative transporter content of the subcellular fraction was determined by quantitative immunoblotting, and the values were expressed as a percent of the transporter content of this fraction in basal cells. The data are the mean ± SEM of five separate experiments. Panel D shows the ratio of the rate of 2-deoxyglucose uptake (percent of maximum; from panel A) to the amount of surface transporter (percent of maximum; panel B) plotted against time of exposure to 1 μM insulin.

Results and Discussion

Time Course for Stimulation of Hexose Transport by Insulin. Figure 1A shows the time course of insulin stimulation of hexose transport in 3T3-L1 adipocytes at 27 °C, as measured by the uptake of 2-deoxyglucose for a 1-min period after exposure of the cells to 1 μM insulin for various times. The half-time for stimulation was 9.5 min, and the fully activated cells exhibited an 8.3-fold increase in uptake rate. The temperature of 27 °C was chosen in order to slow the rate sufficiently to allow accurate comparison of the various time courses (see below). At 37 °C the half-time for stimulation is ~3 min (data not shown). The uptake of 2-deoxyglucose

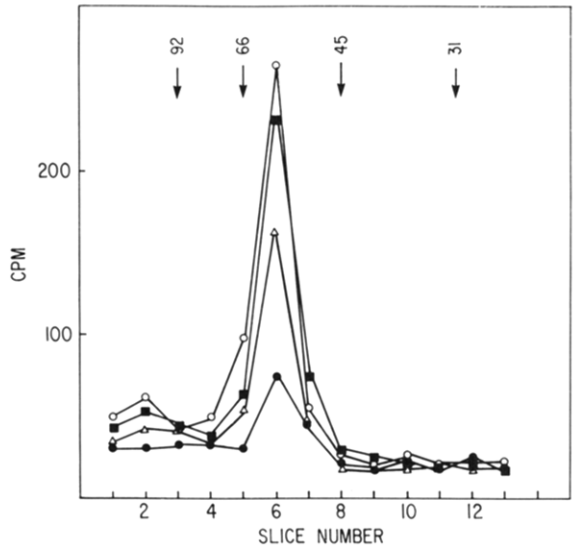

FIGURE 2: Insulin-stimulated increase in glucose transporters at the cell surface. Transporters at the surface of basal (unstimulated) cells (●) and cells exposed to 1 μM insulin for 4 (△), 10 (■) and 30 min (○) were labeled as described under Materials and Methods. Shown are the counts in each gel slice upon electrophoresis of the immunoprecipitate. Quantitative immunoblotting confirmed that the total transporter content of each immunoprecipitate was the same (data not shown).

consists of both transport across the plasma membrane and subsequent phosphorylation of the hexose by hexokinase (Wohlheuter & Plagemann, 1980). Although a previous study has shown that transport is the rate-limiting step at 37 °C (Frost & Lane, 1985), it was important to verify that this was the case under the assay conditions employed in this study. Transport alone was assayed by measuring the uptake of the nonmetabolizable hexose 3-*O*-methylglucose (Wohlheuter & Plagemann, 1980) for a 30-s period after various times of exposure to insulin. With this assay, the half-time for stimulation was 12 min (data not shown). Although this value is slightly longer than that of 9.5 min, the two values are probably not significantly different. Thus, the half-time for stimulation is ~10 min.

Time Course for Increase in Cell Surface Glucose Transporters in Response to Insulin. In order to follow the insulin-induced increase in glucose transporters at the cell surface, we employed a method recently developed in this laboratory in which the oligosaccharide chain(s) of surface proteins, including the transporter, are labeled by treatment of the cells with galactose oxidase and then [^{3}H]borohydride at 4 °C (Calderhead & Lienhard, 1988). Subsequently, the transporters are isolated by immunoprecipitation from a detergent lysate of the cells. Following electrophoresis, the counts associated with the transporter provide a measure of the relative amount of surface transporters. Figure 2 shows data from a representative experiment of this type. The gel shows a single peak of radioactivity at the position where the transporter migrates (M_r 55 000). The magnitude of this peak is almost maximal in cells exposed to insulin for 10 min. The results derived from four separate experiments of this type are summarized in Figure 1B. It is evident that upon exposure to insulin the insertion of additional transporters into the cell surface, as measured by this assay, occurred with a half-time of 4 min. Moreover, the fully stimulated adipocytes possessed only a 2.6-fold larger amount of surface transporters, as compared to an 8-fold increase in the rate of transport. Previously, it was found by these methods that after completion of the insulin stimulation at 37 °C (15 min of exposure) the surface transporters increased 2.6-fold while the transport rate was elevated 11-fold (Calderhead & Lienhard, 1988).

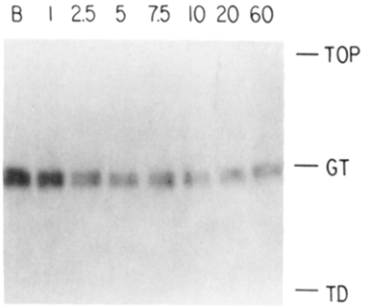

FIGURE 3: Insulin-induced translocation of transporters from the intracellular pool. Cells were stimulated with 1 μM insulin for different times, immediately homogenized, and fractionated as described under Materials and Methods. Quantitative immunoblotting was used to assay the transporter content of a subcellular fraction containing the intracellular, insulin-responsive transporters. An immunoblot of a representative experiment is shown; the time of exposure to insulin (minutes) is given above each lane; B refers to basal (untreated) cells. The counts per minute (cpm) in the transporter band of each lane, after correction for a background of 225 cpm, were as follows: basal 989, 1 min 889, 2.5 min 825, 5 min 733, 7.5 min 668, 10 min 653, 20 min 663, and 60 min 635 cpm. Each lane contained the protein from 2.8% of the supernatant from a 35-mm plate (~10 μg).

Because the surface-labeling procedure involved rapid chilling of the adipocytes to 4 °C and subsequent manipulation at this temperature for 1.5 h after the insulin exposure at 27 °C for various periods (see Materials and Methods), it was necessary to determine whether the shorter half-time was simply due to the continued, but slow action of insulin at 4 °C. To test this possibility, cells were treated with insulin at 27 °C for different times, exactly as in Figure 1A,B. The cells were then washed with ice-cold phosphate buffer and maintained in this buffer at 4 °C for 1 h, in a procedure analogous to that for the surface labeling, except that galactose oxidase and $NaBH_4$ were omitted (see legend of Figure 1B). Finally, the uptake of 50 μM 2-deoxyglucose at 4 °C for 30 min (a period over which it is linear at this temperature) was assayed. The half-time for insulin stimulation of transport determined in this way was 9 min, and the maximal stimulation was 9-fold (data not shown). These values are the same as those obtained upon immediate assay of the transport rate at 27 °C (Figure 1A). Thus, chilling of the cells immediately prevented the further stimulation of transport and, by inference, the increase in cell surface transporters.

Time Course for Decrease in Intracellular Glucose Transporters in Response to Insulin. A subcellular fraction that contains the insulin-responsive intracellular transporters and is largely free of plasma membrane can be readily prepared from 3T3-L1 adipocytes by homogenization and a single centrifugation step (see Materials and Methods). The time course for the translocation of transporters from this fraction, measured under the same conditions as the other two time courses, was determined by quantitative immunoblotting. A representative experiment is presented in Figure 3. In this experiment, the content of intracellular transporters decreased by 34%, with a half-time of ~3 min. Figure 1C summarizes the data from five separate experiments of this type. Insulin caused a 30% reduction in the intracellular transporters with a half-time of 4 min. This finding shows that there is no significant lag between the disappearance of intracellular transporters and the appearance of the additional transporters in the plasma membrane.

Conclusions. This study provides further support for the hypothesis that there is a second mechanism contributing to

the stimulation of hexose transport besides the well-established translocation of transporters from an intracellular site to the plasma membrane (see the introduction). First, the time required to reach the maximal transport rate was considerably longer than the time required for disappearance of the transporters from the intracellular membranes and for their appearance in the plasma membrane. Second, at 27 °C the stimulation of transport (8-fold) was 3 times larger than the stimulation of surface transporters (2.6-fold). The entire effect of insulin on hexose transport in 3T3-L1 adipocytes at 37 °C, as measured by 2-deoxyglucose uptake, has been shown to be on V_{max} (Frost & Lane, 1985). Since V_{max} is a composite term consisting of both a group of rate constants defining the intrinsic activity of the transporter (the turnover number) and the total amount of functional transporters (Gorga & Lienhard, 1984), the most likely basis for this second mechanism is an increase in the intrinsic activity of the transporters. On the assumption that the second mechanism of insulin stimulation is an increase in the intrinsic activity of the plasma membrane transporters, the characteristics of this effect are evident in Figure 1D, which presents the relative intrinsic activity (ratio of transport activity in Figure 1A to surface transporters in Figure 1B) as a function of time. The intrinsic activity increased 3.3-fold with a half-time of 11 min. By comparison with Figure 1B, it is evident that this activation develops about one-third as rapidly as does translocation and contributes slightly more than translocation to the overall stimulation at 27 °C. Thus, this second mechanism is the rate-limiting step in the stimulation of hexose transport by insulin, and the putative increase in the intrinsic activity of the transporters occurs after they have translocated to the plasma membrane. The molecular basis for this second mechanism remains to be elucidated.

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Registry No. Insulin, 9004-10-8; D-glucose, 50-99-7; D-2-deoxyglucose, 154-17-6.

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