

Transfer of secretory proteins from the endoplasmic reticulum to the Golgi apparatus: Discrimination between homologous and heterologous transfer in intact heterokaryons

(secretion/transition vesicles/cell fusion/immunofluorescence microscopy)

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ABSTRACT To examine aspects of the transfer of secretory proteins from the endoplasmic reticulum to the Golgi apparatus *in situ*, heterokaryons were formed between Hep G2 human hepatoma cells and WI-38 human fibroblasts. The cells were appropriately treated with cycloheximide before fusion, which emptied them of their respective secretory proteins, serum albumin for the Hep G2 cells and procollagen I for the WI-38 cells. After fusion was complete, the cycloheximide was washed out, protein synthesis was resumed, and the rates of reappearance of serum albumin and procollagen I in the two separated Golgi apparatuses within each heterokaryon were followed by immunofluorescence microscopy. Serum albumin was found to always reappear first in the Golgi apparatus contributed by the Hep G2 half of the heterokaryon, and procollagen I in the Golgi apparatus of the WI-38 half. These results suggest that the endoplasmic reticulum-to-Golgi apparatus transfer *in situ* is not simply a stochastic process but is either spatially restricted or exhibits cell-type specificity or both.

In this paper, we are concerned with that part of the intracellular pathway for secretion that involves transfer from the endoplasmic reticulum (ER) to the Golgi apparatus. This transfer is thought to occur via transition vesicles that bud off the ER and then fuse at one side (the cis-face) of a stack of Golgi apparatus saccules (cf. refs. 1–3). The biochemistry and genetics of the transfer process are being addressed in several laboratories, and a number of protein components that are implicated have been identified (for review, see ref. 4). In addition to the biochemistry, however, there are many structural aspects of the transfer process to be considered. The ER is a ramified membrane-bounded structure usually dispersed throughout the cytoplasm, whereas the Golgi apparatus is often a compact organelle confined near and to one side of the cell nucleus. Does the transfer process involve budding of the transition vesicles from the ER at random regions throughout the cytoplasm and then diffusion of the transition vesicles to the cis-face of a Golgi apparatus stack? Or is the transfer process more restricted spatially and structurally (see *Discussion*)? To help distinguish between these two broad alternatives, the following experiments were devised and carried out.

Heterokaryons were formed in culture between two human cells secreting different proteins. Before cell fusion, the cells were treated with cycloheximide to empty them of their respective secretory proteins (5); shortly after fusion, the cycloheximide was washed out, and protein synthesis was reinitiated in the heterokaryons. By immunofluorescence microscopic analysis of the heterokaryons for the distributions of the appropriate secretory proteins, performed at

different times shortly after protein synthesis was resumed, we determined rates of reappearance of the secretory proteins in the two separated Golgi apparatuses contributed by each of the two cells forming the heterokaryon. If the process of transition vesicles budding from the ER occurs randomly throughout the cytoplasm, and the transfer of the transition vesicles to the Golgi apparatus occurs by simple diffusion, we reasoned that a secretory protein might reappear simultaneously in both Golgi apparatuses of a heterokaryon. We report here that, on the contrary, a secretory protein always reappeared first in that Golgi apparatus for which it was homologous, and only after a significant delay, in the heterologous Golgi apparatus of the heterokaryon. Possible interpretations of these results are discussed. A preliminary account of these studies has been published (6).

MATERIALS AND METHODS

Cells and Heterokaryons. Hep G2 human hepatoma cells (0.2×10^6) and WI-38 human fibroblasts (0.15×10^6) in 5 ml of medium (Dulbecco's modified Eagle's medium/10% fetal calf serum) were coplated on 12 coverslips in a 6-cm dish at 37°C 15–18 hr before fusion. Under these conditions, the cells spread and began to overlap. At 2.5 hr before fusion, the culture fluid was replaced with one containing cycloheximide at 80 µg/ml of medium. Just before fusion, the mixed monolayer was treated with trypsin (100 µg/ml) and collagenase (100 µg/ml) in serum-free medium containing cycloheximide for 3 min at room temperature. This treatment promoted the efficiency of fusion. The enzyme solution was then washed away and replaced with a droplet of 50% polyethylene glycol (PEG 4000) in serum-free medium containing cycloheximide. After 10–15 sec at 37°C, the polyethylene glycol was carefully washed out with medium containing cycloheximide. Twenty minutes later, the cycloheximide was rapidly and thoroughly washed out with fresh medium at 37°C, and protein synthesis was reinitiated.

Immunofluorescence Microscopy. At different times after protein synthesis was reinitiated, individual coverslips were fixed with 3% formaldehyde for 10 min at room temperature and permeabilized with 1% Triton X-100 for 3–5 min at room temperature. The cell mixtures were then labeled with either (i) affinity-purified polyclonal rabbit antibodies to human serum albumin plus monoclonal mouse antibodies (Developmental Studies Hybridoma Bank, The Johns Hopkins University, Baltimore) to human procollagen I; or (ii) the rabbit antibody to serum albumin. In the first case, the primary labeling was followed by a mixture of rhodamine-labeled

Abbreviations: ER, endoplasmic reticulum; FI-WGA, fluorescein-conjugated wheat germ agglutinin.

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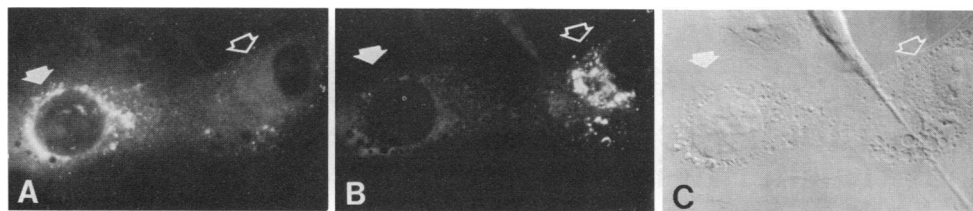


FIG. 1. Heterokaryon formed between Hep G2 hepatoma cell and WI-38 fibroblast in their steady states just before fusion, doubly immunofluorescently labeled for serum albumin and procollagen I. (A) Immunolabeled for serum albumin, the Hep G2 Golgi apparatus is labeled (filled arrow) but not the WI-38 Golgi apparatus (open arrow). (B) Immunolabeled for procollagen I, labeling pattern is the converse. (C) The same heterokaryon viewed in Nomarski optics. (For magnification, see Fig. 4.)

F(ab')₂ fragments of goat antibodies to rabbit IgG (Jackson ImmunoResearch) and biotin-labeled F(ab')₂ fragments of goat antibodies to mouse IgG (Cooper Biomedical), and finally fluorescein-conjugated streptavidin (EY Laboratories). In the second case, the primary labeling was followed by rhodamine-labeled F(ab')₂ fragments of goat antibodies to rabbit IgG plus fluorescein-conjugated wheat germ agglutinin (Fl-WGA) (Calbiochem). The Fl-WGA labeling allowed visualization of both Golgi apparatuses in the heterokaryon. Immunofluorescence microscopy was carried out with a Zeiss photoscope III epifluorescence instrument equipped with Nomarski optics. Observations by Nomarski optics generally allowed easy morphological discrimination between the flattened extended WI-38 half and the more rounded Hep G2 half of the heterokaryon.

RESULTS

A variety of experiments and controls with different types of heterokaryons were carried out in this study, the bulk of

which will be reported elsewhere. Here we present the results of one set of experiments with heterokaryons produced by the fusion of Hep G2 human hepatoma cells, processing the secretory protein serum albumin, with WI-38 human fibroblasts, processing the protein procollagen I. In control experiments carried out with heterokaryons made from the two cells in their steady states in culture, shortly after cell fusion serum albumin was readily detectable only in that Golgi apparatus contributed by the Hep G2 portion of a heterokaryon (filled arrow, Fig. 1A), whereas procollagen I was seen only in the Golgi apparatus from the fibroblast (open arrow, Fig. 1B). Similar fusion experiments showed that the extent of fusion as observed morphologically (as in Fig. 1C) was ≈80% complete by 10 min and 100% complete by 15 min, after polyethylene glycol addition.

In the main series of experiments, cells were treated with cycloheximide for 2.5 hr before fusion, polyethylene glycol was then added in the presence of cycloheximide, and 20 min later (after fusion was complete) the cycloheximide was

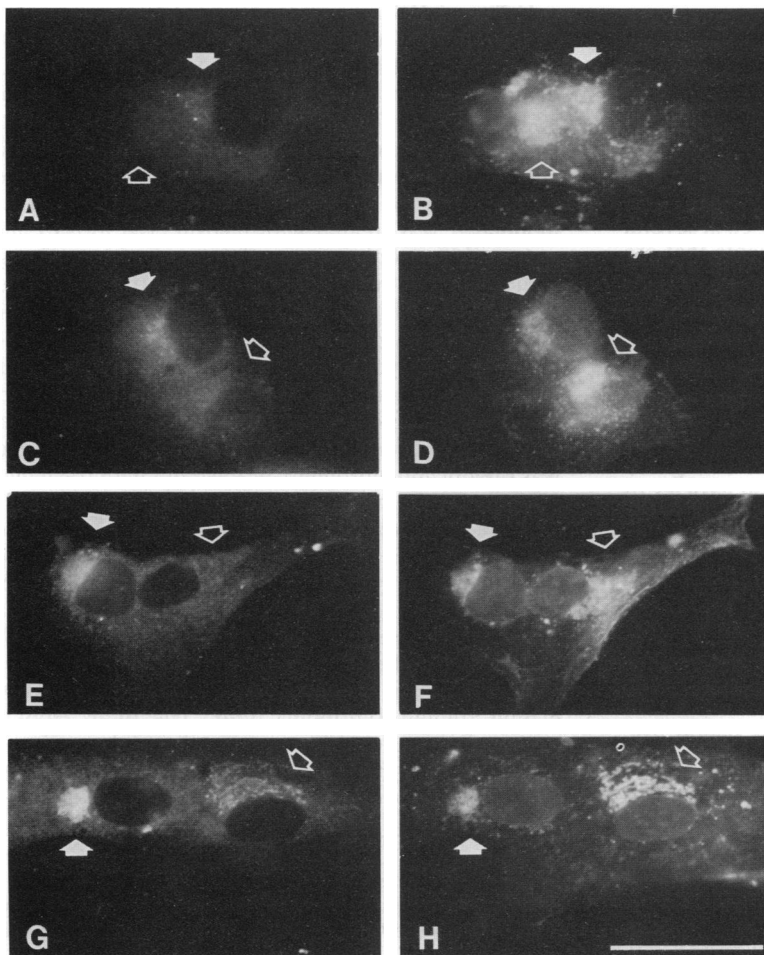


FIG. 2. Similar heterokaryons as in Fig. 1, formed from cells treated with cycloheximide for 2.5 hr before fusion, then washed free of cycloheximide 20 min after fusion was initiated, and doubly labeled for serum albumin (A, C, E, and G) or with Fl-WGA to detect both Golgi apparatuses (B, D, F, and H, respectively) at the following times after cycloheximide was washed out: A and B, 0 time; C and D, 10 min; E and F, 20 min; G and H, 30 min. The filled arrow designates the Hep G2 Golgi apparatus, and the open arrow designates WI-38 Golgi apparatus of the heterokaryon. (Bar in H = 50 μm.)

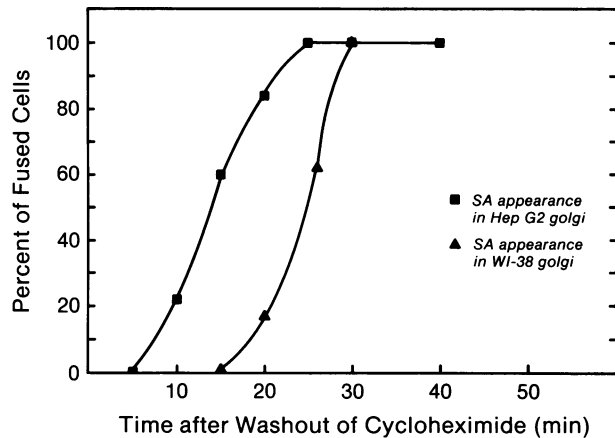


FIG. 3. Reappearance of serum albumin (SA) in the two Golgi apparatuses inside Hep G2-WI-38 heterokaryons with time after protein synthesis was reinitiated. Results of three independent sets of experiments, in each of which at least 30 heterokaryons were scored at each time point, are summarized.

rapidly washed out at 37°C to reinitiate protein synthesis. This reinitiation marked time zero in all following experiments. At time zero, serum albumin (Fig. 2A) was no longer detectable inside the heterokaryons (compare with Fig. 1), as a result of the cycloheximide treatment before cell fusion. In the following figures, the Hep G2 Golgi apparatus is always marked by a filled arrow, and the WI-38 Golgi apparatus is marked by an open one. By 10 min, serum albumin was now detectable and was mainly diffusely distributed, presumably in the ER (4); this diffuse labeling was dispersed throughout the entire heterokaryon (Fig. 2C). At this time, of the two separated Golgi apparatuses of the heterokaryon (detected by simultaneous labeling with FI-WGA, Fig. 2D), the Golgi apparatus derived from the Hep G2 cell (filled arrows, Fig. 2C and D) showed a slightly enhanced labeling for serum albumin, but not the Golgi apparatus derived from the WI-38 cell (open arrows, Fig. 2C and D). By 20 min, the concentration of serum albumin in the Hep G2 Golgi apparatus (filled arrow, Fig. 2E) was now at steady-state level, but still no serum albumin was detectable in the WI-38 Golgi apparatus (open arrows, Fig. 2E and F). By 30 min, serum albumin was found concentrated in both Golgi apparatuses (Fig. 2G). A large number of such experiments all gave closely similar results and yielded the data plotted in Fig. 3. These results show that it took an average of ≈ 14 min after protein

synthesis was reinitiated for serum albumin to reach steady-state levels in the Hep G2 Golgi apparatus, and then another 10–12 min before serum albumin occupied the WI-38 Golgi apparatus in these heterokaryons.

When similar heterokaryons were examined by double immunofluorescence labeling for procollagen I and serum albumin, after cycloheximide treatment, procollagen I was not detectable in the heterokaryons (Fig. 4B). After reinitiation of protein synthesis the intracellular increase in procollagen I occurred at a slower rate than for serum albumin. At 60 min after reinitiation, by which time serum albumin had fully occupied both Golgi apparatuses (Fig. 4D; see Fig. 2G), procollagen I was found only in the WI-38 Golgi apparatus (Fig. 4E). By ≈ 90 min, migration of the two Golgi apparatuses into overlapping positions in the heterokaryon rendered them no longer distinguishable. Before this termination point of the experiment, procollagen I was never detected in the Hep G2 Golgi apparatus.

DISCUSSION

These experiments demonstrate that in heterokaryons made between two human cells, the transfer of a secretory protein occurred more rapidly between homologous ER and Golgi apparatus elements (that is, both derived from only one of the two fused cells) than between heterologous ones. Certain trivial explanations of these results can be eliminated.

(i) The preference for homologous transfer was not due to the presence of residual ribosome-attached nascent polypeptide chains associated with the homologous ER in the cycloheximide-treated heterokaryons because the same time lag between the appearance of serum albumin in the homologous and heterologous Golgi apparatuses of the heterokaryons was observed if, instead of cycloheximide, puromycin was used in these experiments (data not shown). On the other hand, the possibility that the particular mRNA molecules involved in this study, once synthesized in a cell, remained bound to the homologous ER via its associated cytoskeleton (cf. refs. 7 and 8), may be an important factor in explaining our experimental results.

(ii) The time lag was not attributable to shorter diffusional distances between homologous ER and Golgi apparatus elements as compared with heterologous ones in the heterokaryons. It is important to note that, after fusion, the ER elements of the two contributing cells appeared to intermingle rapidly, because the ER labeling for serum albumin observed within a few minutes after reinitiation of protein synthesis

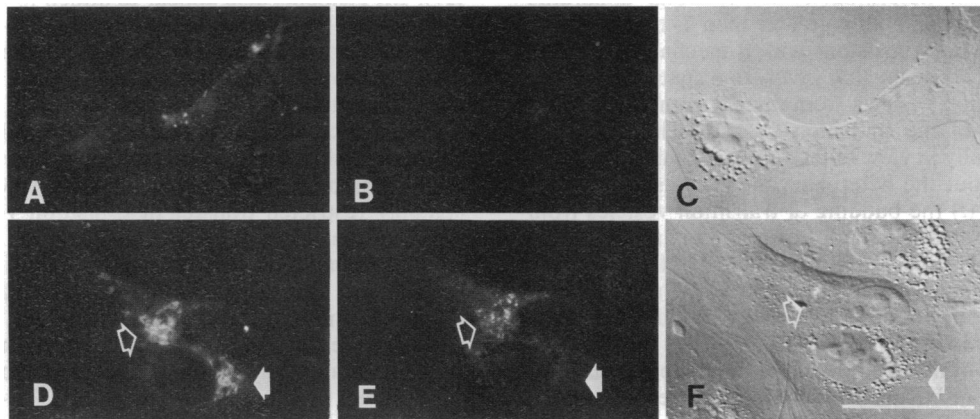


FIG. 4. Similar heterokaryons as in Fig. 1, formed from cells treated with cycloheximide for 2.5 hr before fusion and then washed free of cycloheximide 20 min after fusion was initiated, were doubly labeled for serum albumin (A and D) and procollagen I (B and E), respectively, at the following times after cycloheximide was washed out: A–C, 0 time; D–F, 60 min. In D, E, and F, the filled arrow designates the Hep G2 Golgi apparatus, and the open arrow designates the WI-38 Golgi apparatus of the heterokaryon. C and F are the respective Nomarski pictures. (Bar in F = 50 μ m, the same magnification as for Fig. 1.)

was spread throughout the heterokaryon (cf. Fig. 2C). This suggests that at least grossly each of the two ER networks in the heterokaryon was similarly distributed with respect to each of the two Golgi apparatuses.

Furthermore, the time lag observed between homologous and heterologous transfer was independent of the distance of separation of the two Golgi apparatuses from one another in any given heterokaryon (compare, for example, Fig. 2C and D with Fig. 2G and H). If the heterologous transfer were slower because of longer diffusion paths of the transition vesicles through the cytoskeletal matrix, one would have expected, on the contrary, that the time lag in the appearance of serum albumin in the heterologous compared with the homologous Golgi apparatuses would have varied with the distance between the two Golgi apparatuses.

(iii) The observations were not due to peculiarities of the particular system under study. We have obtained closely similar results with Hep G2-WI-38 heterokaryons, detecting the reappearance of retinol-binding protein made by the Hep G2 moiety, and with Hep G2-HeLa cell heterokaryons, detecting the reappearance of serum albumin (unpublished results). Furthermore, there was nothing peculiar about the characteristics of serum albumin reappearance in the Hep G2-WI-38 heterokaryons used in this study. These were the same as observed for serum albumin reappearance with cycloheximide-treated unfused Hep G2 cells and with homokaryons made by polyethylene glycol fusion of cycloheximide-treated Hep G2 cells (unpublished results).

We conclude that the observation of a faster rate of reappearance of a secretory protein in the homologous compared with the heterologous Golgi apparatus of a heterokaryon requires a nontrivial explanation.

Before discussing such possible explanations, it should be recognized that the appearance of a secretory protein in the heterologous Golgi apparatus in a heterokaryon could arise via several possible routes: these include a direct, albeit delayed, transfer from the homologous ER to the heterologous Golgi apparatus, or an indirect transfer from the homologous Golgi apparatus (only after it was occupied by the secretory protein) to the heterologous Golgi apparatus. Such inter-Golgi apparatus transfers in heterokaryons *in situ* have been reported (9). If the former possibility were the correct one, then we estimate from the data that the ER-to-Golgi apparatus transfer occurred at a rate 2 to 4 times greater between homologous than between heterologous elements. If, however, the heterologous Golgi apparatus was occupied mainly by transfer from the homologous Golgi apparatus and not directly from the homologous ER, then the relative rates of homologous vs. heterologous ER-to-Golgi apparatus transfer could be considerably greater than 2- to 4-fold.

Our results are not consistent with a mechanism of ER-to-Golgi apparatus transfer that involves a spatially random budding of transition vesicles from the ER combined with a stochastic fusion of the transition vesicles with the Golgi apparatus, especially in view of the rapid intermingling of the two ERs throughout the heterokaryon. Our results suggest rather that either (i) the budding of transition vesicles from the ER may not be spatially random; or (ii) there may be some chemical specificity involved in the transfer of the transition vesicles of one human cell type to the Golgi apparatus of another; or (iii) both possibilities coexist. We discuss these possibilities in turn.

(i) The budding of transition vesicles from the ER may not be spatially random but may be confined to regions where elements of the ER (transitional elements, cf. refs. 1 and 2) are situated proximal to the cis-face of a Golgi stack. This is the clear message, for example, of a spectacular electron micrograph taken of the protozoan, *Trebonema vulgare* (figure 19 in ref. 10). This figure shows a region of the outer nuclear membrane (part of the ER), located directly opposite

to the cis-face of a Golgi stack, that was apparently in the process of actively budding off a number of transition vesicles, whereas immediately adjacent portions of the same nuclear membrane that were not apposed to the Golgi apparatus were quiescent. Some precisely demarcated structural relationship of portions of the ER that are sites for transition vesicle budding, with the cis-face of a Golgi stack, is strongly implied by these findings. If this suggestion has general validity, it may explain several diverse observations. A critical role for a structural relationship between elements of the ER and Golgi apparatus could explain, for example, why for *in vitro* studies of the transfer from the ER to the Golgi apparatus to be successful, cells must not be excessively fragmented but, instead, must be retained structurally nearly intact after permeabilization (4). [On the other hand, this is not so for Golgi-to-Golgi transfer, which occurs efficiently when subcellular fractions extracted from cells are used (11).] Furthermore, inhibition of the transfer of secretory proteins from the ER to the Golgi apparatus that is known to occur during mitosis might be explained were certain critical ER-Golgi apparatus structural relationships disrupted as Golgi elements become fragmented during mitosis (12). For a structural relationship between elements of the ER and Golgi apparatus to explain our results, however, that relationship must exist between the homologous elements in the heterokaryon but must be slow to form or be unable to form between heterologous ones.

What such proposed structural relationships between elements of the ER and the cis-face of the Golgi apparatus might involve can only be speculated about. There have been numerous reports of electron micrographs showing apparently direct connections between the ER and Golgi apparatus (cf. refs. 13 and 14), but the significance of such connections is controversial. A structural relationship need not, however, involve a direct connection. Such a relationship could consist, for example, of some localized microtubules that are essential to mediate ER→Golgi apparatus traffic. It is known that the Golgi apparatus is closely associated with microtubules. In interphase cells, the microtubule-organizing center, the single compact cytoplasmic region from which all microtubules emanate, closely overlaps the region occupied by the Golgi apparatus (cf. ref. 15). Therefore, a dense mat of microtubules stemming from the microtubule-organizing center pervades the region occupied by the Golgi apparatus. Furthermore, vesicular elements of the Golgi apparatus appear to track on microtubules as they reassemble after their nocodazole-induced dispersion (16), and when the Golgi apparatus is disassembled upon treatment with brefeldin A (17). ER elements also appear to attach to microtubules and elongate by locomotion along microtubule tracks (18). It is, therefore, possible that ER-to-Golgi apparatus traffic is mediated by way of tubular extensions of the ER that occur only along microtubule tracks, arising from ER elements located close to the cis-face of the Golgi apparatus. Transition vesicle budding could be specifically induced only at these tubular extensions of the ER. These processes would result in a spatially restricted transfer of transition vesicles from elements of the ER to the Golgi apparatus.

(ii) Alternatively, the budding of transition vesicles from the ER may occur in a spatially random manner throughout the cytoplasm, but the mechanisms of the formation of the transition vesicles and/or their fusion at the cis-face of the Golgi apparatus may involve protein components distinctive for different cell types. For example, GTP-binding proteins have been implicated in the process of ER-to-Golgi transfer (cf. ref. 4); such proteins are members of a superfamily of *ras*-related proteins, and this family could be sufficiently diverse that they exhibit some degree of cell-type specificity for a given process like ER-to-Golgi transfer. Such specificity might be involved in the different rates of homologous

ER-to-Golgi transfer of serum albumin and of procollagen I (Fig. 4).

(iii) Finally, conceivably both of the above-mentioned possibilities coexist—namely, there is a spatial and structural restriction to the process of transition vesicle budding from the ER, and there also are distinctive cell-type components involved in the transfer of the transition vesicle to the Golgi apparatus. The combined effects of these two nonrandom events could result in the homologous ER-to-Golgi transfer occurring more rapidly than the heterologous one.

It is evident that the observations made in this report suggest a number of significant directions for further investigation.

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