Dissociation and re-assembly of the endoplasmic reticulum in live cells

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Summary

The endoplasmic reticulum (ER) of a typical interphase 3T3 fibroblast consists of a compact perinuclear arrangement of cisternae and lamellae which can be observed by immunofluorescence with anti-endoplasmin. During mitosis the reticulum dissociates into small fragments from which it appears to re-assemble in the daughter cells. When interphase 3T3 cells are exposed to calcium ionophores, but not other ionophores, there is a similar dissociation of the ER into small uniform fragments, which are dispersed throughout the cytoplasm. Electron microscopy shows that the fragments consist of small vesicular structures and that essentially all of the rough ER except the nuclear envelope is dissociated. The dissociation of the ER by calcium ionophore is a relatively specific process since other organelles and supramolecular assemblies remain unaffected. When cells with dissociated ER are returned to normal medium, there is a rapid reassembly of the fragments into the continuous reticulum. In a proportion of the cells it is possible to observe linear arrays of the fragments, which probably represent intermediates in the re-assembly process. These observations demonstrate that the ER in interphase 3T3 cells can be dissociated into, and re-assembled from, small fragments. Re-assembly of the ER from the fragments is dependent on the presence of millimolar levels of calcium in the external medium. In the presence of calcium, re-assembly is inhibited by the calcium channel blocker, verapamil. Thus calcium ions appear to play an important role in ER structure and assembly.

Key words: ER dissociation, ER assembly, ER subunits, reticulosomes, calcium ionophores, ER calcium.

Introduction

The endoplasmic reticulum (ER) consists of a complex system of tubules and lamellae, which can assume a variety of morphologies in different cells (Krstic, 1979). In spite of the fact that it was first described in the 1890s (see review by Fawcett, 1981) and subsequently formally characterized in the 1940s (Porter et al. 1945), it remains relatively poorly understood in terms of its basic structure, synthesis and assembly. An issue of particular importance concerns the mechanism of synthesis and assembly of the continuous reticulum. It has been observed in at least some cells that the ER does not remain a continuous structure throughout the cell cycle, and appears to fragment at the onset of mitosis and re-assemble from the fragments after cell division (Zeligs & Wollman, 1979). This suggests that in at least some cases cells are able to form the continuous reticulum by a process of assembly from pre-formed subunits. However, analysis of this process is intrinsically difficult in mitotic cells because of their scarcity in a population of cells. Although techniques have been developed for the generation of enriched populations of mitotic cells (Zieve et al. 1980) these involve the use of microtubule-dissociating agents. Alternatively, systems are being developed for the analysis of ER assembly in cell-free conditions (Dabora & Sheetz, 1987). However, these need to be complemented with studies on the process in vivo at least to ensure that the in vitro systems are representative of the process in living cells. It would therefore be valuable to develop a system that lends itself to the analysis of the dissociation and re-assembly of the ER in live interphase cells. In this context it is noteworthy that ER dissociation does occur in non-mitotic cells (Trump et al. 1976; Zeligs et al. 1975) exposed to a variety of non-lethal stresses and the common factor in such cases appears to be alterations in the calcium balances in the cells (Trump et al. 1981). These effects can be simulated by treating non-mitotic
cells with calcium ionophores (Schanne et al. 1979). It is worth noting that calcium ionophores also have specific effects on the composition of the ER since they cause the specific over-expression of several calcium-binding ER proteins (Resendez et al. 1985; Macer & Koch, 1988).

In this study we show that it is possible to simulate the dissociation-assembly cycle of the ER, which occurs in 3T3 fibroblasts during mitosis, by subjecting cells to a brief treatment with calcium ionophore. The ER was generally visualized by immunofluorescence using antibodies specific for the ER protein endoplasmic (Koch et al. 1986). Immunofluorescence analysis was the preferred procedure for routine examination of ER morphology because it provides an overall picture of the ER. However, when necessary such studies were complemented with appropriate electron-microscopic examination of samples. These studies demonstrate that the ER can be re-assembled from fragments in vitro and draw attention to the central importance of calcium ions in the structure and re-assembly of the continuous reticulum.

Materials and methods

All ionophores, protein synthesis inhibitors and tunicamycin were purchased from Sigma. Low-calcium medium was formulated by Gibco and was a kind gift from M. Hanley. The medium was standard Dulbecco's modified Eagle's minimum medium without any of the normal calcium salts. It was estimated that the calcium concentration was less than 1 µM.

Cells were maintained in either RPMI 1640 medium (NIH-3T3, P815) or Dulbecco's modified Eagle's medium (MOPC 315) with 10% foetal calf serum.

For standard dissociation and re-assembly experiments glass slides were coated with polylysine and trypsinized NIH-3T3 cells in phosphate-buffered saline (PBS) allowed to settle and attach to the slides. Slides were then immersed in complete RPMI 1640 medium and incubated at 37°C to permit spreading to proceed. After 16 h cells were used for the appropriate treatment.

Cells were prepared for immunofluorescence with anti-endoplasmic by immersion of slides in 3-5% formaldehyde/PBS for 5 min followed by 5 min in 1% Nonidet P40 (NP40)/PBS. After washing in PBS, rabbit anti-endoplasmic (Koch et al. 1986) was applied for 5 min, followed by FITC-goat anti-rabbit immunoglobulin (Ig) for 5 min. Slides were washed and mounted in 50% glycerol/PBS with 0.2% p-phenylenediamine as anti-fade. Cells were viewed by conventional epifluorescence microscopy or by confocal fluorescence microscopy, as described previously (Koch et al. 1987).

Electron microscopy was carried out as described previously (Koch et al. 1988). Briefly, cells were fixed in situ in glutaraldehyde, scraped off with a rubber policeman, pelleted, treated with osmium-ferricyanide, dehydrated in ethanol and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate. This protocol does not highlight the ribosomes on the rough ER.

Results

Morphology of the ER in interphase murine fibroblasts

Since the study of ER dissociation and re-assembly requires a clear definition of the normal morphology of the cells under consideration, it was necessary to examine ER morphology in murine 3T3 fibroblasts, which was the main system used in this investigation. When 3T3 cells are grown at sub-confluent densities and the ER stained with antibody to endoplasmic, the majority of the cells exhibit the type of morphology seen in Fig. 1. The characteristic feature is that it is quite compact and largely confined to the region surrounding the nucleus. This is emphasized in Fig. 1B by marking the cell outline with the broken line. Typically, >95% of the cells express this type of morphology. A, ×800; B, ×1600.

Fig. 1. Morphology of the ER in interphase 3T3 cells grown at low density. Cells were seeded at such a level as to ensure that they did not form obvious contacts after a 16 h incubation. They were then prepared for immunofluorescence with antibody to endoplasmic. Note the compact perinuclear morphology of the ER which is emphasized in B by marking the cell outline with the broken line. Typically, >95% of the cells express this type of morphology. A, ×800; B, ×1600.
Fig. 2. Ultrastructure of the ER in 3T3 cells. Electron micrographs show that ER is composed of continuous tubules and flattened lamellae. In vertical section (D) these appear to run parallel to the substratum. A, B, ×2600; C, ×5000; D, ×13 000.

Fig. 3. Dissociation and re-assembly of the ER in 3T3 cells during cell division. Cells were prepared for immunofluorescence with anti-endoplasmin and mitotic cells identified by the absence of a nucleus or a nuclear membrane (B). Note the difference in the staining pattern of the ER in the dividing cell where it is punctate and apparently fragmented in contrast to the typical pattern of the interphase cell (A). C shows that the normal morphology has been re-established before the daughter cells have separated. A, B, ×4000; C, ×1600.
membrane (Fig. 2). Examination of many EM images also confirmed that the ER is confined to the perinuclear region (unpublished observations).

**ER morphology during cell division**

During mitosis a significant change in ER morphology was observed. Thus in a field in which >90% of the interphase cells showed the compact perinuclear morphology described above, cells that were undergoing division showed a more punctate pattern of fluorescence upon staining with anti-endoplasmin, suggesting that the ER had fragmented during the process of division (Fig. 3B). This was confirmed by electron microscopy of cells in metaphase, in which the ER was clearly disassociated into relatively small fragments, which were dispersed throughout the cytoplasm, although largely excluded from the mitotic spindle (Fig. 4). ER re-assembly appears to occur very rapidly after division since the compact continuous cisternal morphology re-appears in the daughter cells even before the latter have become fully separated (Fig. 3C).

These observations suggested that during the normal cycle of 3T3 fibroblasts the ER undergoes dissociation into small fragments which subsequently re-assemble into the continuous reticulum.

**Dissociation of the ER in interphase cells with calcium ionophore**

When 3T3 cells are incubated in medium containing the calcium ionophore, A23187, and the ER examined by immunofluorescence with anti-endoplasmin, the pattern of staining is significantly different from that of the control cells. Instead of the compact pattern seen in the latter (see Fig. 1), the fluorescence is more evenly distributed, generally filling the entire cytoplasm from nuclear to plasma membrane (Fig. 5). The second fea-

![Fig. 4. Ultrastructure of the ER in dividing 3T3 cells. The electron micrograph shows that most of the ER is made up of small fragments dispersed throughout the cytoplasm. A, x15 000; B, x3300.](image-url)
tue of the fluorescence in the cells exposed to ionophore is that it appears to be composed almost entirely of small spots or fragments, rather than the continuous pattern of the control cells. These fragments are so extensively dispersed that they even enter into the projections extending from the cell body. In a typical experiment the dispersed fragmented ER morphology is exhibited by >90% of the cells after treatment with the ionophore.

Electron microscopy of the treated cells confirms that exposure to calcium ionophore causes a dramatic change in the morphology of the endoplasmic reticulum. Thus, instead of the continuous profiles of tubules and lamellae, the ER is composed almost entirely of small fragments (Figs 6, 7). Most of the ER fragments consist of small vesicles, whereas many of the larger fragments consist of structures apparently in the process of dissociating into the small vesicles. It is noteworthy that in a significant proportion of the small fragments the outline is interrupted by tail-like structures (Fig. 7). The tails are much narrower and appear to consist of ER membranes without any luminal space. An interesting feature of the ER fragments generated with calcium ionophore was their apparent uniformity. This was particularly evident when they were extracted from ionophore-treated cells and examined by confocal immunofluorescence microscopy where they displayed a ±7% range in diameter (data not shown).

**Re-assembly of the ER from fragments induced with calcium ionophore**

When cells that have had their ER dissociated with calcium ionophore are returned to normal medium, there is a rapid change in the pattern of fluorescence obtained with anti-endoplasmic. The punctate pattern associated with the fragmented ER disappears and the staining assumes the continuous pattern normally associated with the organelle. In most cells (>90%) this re-assembly occurs in about 30 min and this period was used in the experiments described below. The exact pattern of the re-assembled ER varies but one significant difference is that the re-assembled ER is usually more evenly dispersed throughout the cytoplasm than the ER in control cells (see Fig. 8). Thus in many of the cells with re-assembled ER the fluorescence staining extends all the way to the cell periphery. Electron microscopy of cells with a re-assembled ER confirmed that the ER in >90% of the cells consisted of a continuous reticulum of tubules and lamellae (unpublished data).

ER re-assembly from the fragments occurs very rapidly after removal from the ionophore. Within 5 min it is apparent that significant areas have re-assembled into continuous tubules or lamellae and after 30 min most of the cells show the tubular-lamellar configuration. In some cells (Fig. 9) it is apparent that the fragments become aligned in distinct linear arrays, indicating that re-formation of the continuous ER from the fragments occurs by direct re-assembly.

**Involvement of calcium ions in ER dissociation and re-assembly**

Dissociation of the ER into fragments also occurs with comparable efficiency when cells are treated with ionomycin, which is reputed to be a more specific calcium ionophore than A23187. In contrast, ionophores such as valinomycin, nigericin, and gramicidin with specificity towards other ions have undetectable effects on ER morphology under similar conditions (Table 1). These observations suggest that calcium ions are particularly important in the maintenance of ER morphology and integrity.

Calcium ions are necessary for the restoration of ER morphology from the dissociated state. This can be demonstrated directly by carrying out the re-assembly step in a low calcium (<1 μM) medium. In the absence of added calcium significant re-assembly (i.e. in >10% of the cells) is not observed in this medium. However, the addition of 1 mM-calcium results in the re-assembly of the continuous reticulum in >90% of the cells, providing direct evidence that extracellular calcium is required for re-assembly (Fig. 10). Further support for this con-
Fig. 6. Ultrastructure of the ER in 3T3 cells treated with calcium ionophore. The electron micrographs of the A23187-treated cells (see Fig. 5) show that the ER is largely composed of small vesicular fragments. A, ×3200; B, ×6400; C, ×9700; D, ×32000; E, ×6400.
Table 1. Effect of various treatments on the morphology of the endoplasmic reticulum in 3T3 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ER morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>&gt;90% perinuclear lamellae/tubules</td>
</tr>
<tr>
<td>5 µM-A23187</td>
<td>&gt;90% fragmentation</td>
</tr>
<tr>
<td>5 µM-ionomycin</td>
<td>&gt;90% fragmentation</td>
</tr>
<tr>
<td>5 µM-gramicidin</td>
<td>No change from controls</td>
</tr>
<tr>
<td>5 µM-nigericin</td>
<td>No change from controls</td>
</tr>
<tr>
<td>5 µM-monensin</td>
<td>Some fragmentation (&lt;25%)</td>
</tr>
<tr>
<td>2 µM-valinomycin</td>
<td>No change from controls</td>
</tr>
<tr>
<td>10 µM-colchicine</td>
<td>ER dispersed but no fragmentation</td>
</tr>
<tr>
<td>10 µM-nocodazole</td>
<td>Same as colchicine</td>
</tr>
<tr>
<td>10 µM-cytochalasin b</td>
<td>No change from controls</td>
</tr>
<tr>
<td>100 µM-tunicamycin</td>
<td>No change from controls</td>
</tr>
<tr>
<td>100 µM-verapamil</td>
<td>No change from controls</td>
</tr>
<tr>
<td>1 mM-caffeine</td>
<td>No change from controls</td>
</tr>
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</table>

3T3 cells were grown on glass slides and incubated in complete RPMI 1640 medium in the presence of each of the additions shown for 30 min at 37°C. Samples were prepared for immunofluorescence with anti-endoplasmin and the pattern in 50 cells examined. Each cell was scored for a continuous or fragmented pattern of endoplasmin staining and the % cells expressing each pattern calculated.

ER dissociation and assembly

Involvement of cytoskeletal elements in ER re-assembly

The possible involvement of cytoskeletal elements such as microtubules in the re-assembly of the ER from the ionophore-induced fragments was suggested by the observation that the fragments formed linear (beads-on-string) arrays during the re-assembly phase. However, double-labelling experiments on cells exhibiting this configuration of ER with anti-tubulin to display microtubules did not reveal any clear co-linearity between the two systems. Similarly, no obvious association with stress-fibres or intermediate filaments could be observed (data not shown).

Pharmacological studies on this question were inconclusive because many of the agents used to disrupt structures such as microtubules (colchicine, nocodazole, etc.) do have observable effects on the ER (Table 1). However, it should be emphasized that the effect of these agents is not to dissociate the ER into fragments but rather to cause the endoplasmin fluorescence used to
detect the ER to spread throughout the cell. Thus, examination of the effects of such agents on re-assembly were difficult to define and interpret without more extensive analyses on their precise effects on the ER.

**ER dissociation and re-assembly in other cells**

The studies described above were carried out with murine 3T3 fibroblasts. To examine the generality of the major observations some other cells were examined. The ER of calcium ionophore-treated P815 (murine macrophage-type) cells (Smith & Koch, 1987) was also found to dissociate rapidly into fragments similar to those produced in 3T3 cells and to re-assemble into an extensive tubular network upon removal of the ionophore. An exception to the above was the murine plasmacytoma cells MOPC 315, which showed no evidence of ER fragmentation at non-lytic concentrations of calcium ionophore (<2.5 μM). Significantly, such cells also show no evidence of ER dissociation during mitosis (Koch et al. 1987).

**Discussion**

The first objective of this study was to develop a system in which the dissociation and reassembly of the ER thought to occur in many cells during the normal cell cycle (Zeligs & Wollman, 1979) could be examined. The 3T3 fibroblast cells used are widely employed for the examination of a variety of cellular phenomena, so it was particularly relevant to employ these cells. However, it was necessary to examine first the normal morphology of the ER in order to determine its suitability for these studies. For this purpose the ER was displayed with antibodies to the major ER-specific luminal protein, endoplasm (Koch et al. 1986). It is worth emphasizing that endoplasm has only been detected in the rough ER (RER) (Koch et al. 1986 and unpublished data) so the observations described may only apply to this part of the total ER. It was observed during preliminary studies that the RER of 3T3 cells was generally a quite compact structure made up largely of flattened lamellae closely packed around the nucleus. Since this is a particularly convenient arrangement for the studies described here, conditions were developed for optimizing the adoption of this compact, perinuclear morphology in control cells. The crucial factor is that the cells should be grown separate from one another, as at increased density the ER appears rather less compact.

**Fig. 8.** Re-assembly of the continuous reticulum from ER fragments generated in living 3T3 cells with calcium ionophore. Control cells were treated with A23187 to cause ER fragmentation then placed in normal medium for 30 min. Samples were prepared for immunofluorescence with anti-endoplasm. A, control cells; B, ionophore-treated cells; C, ionophore-treated cells re-incubated in normal medium. Note the re-formation of the continuous reticulum after return of the cells to normal medium. It is commonly observed that the re-formed reticulum is not as tightly packed around the nucleus as that in control cells and therefore forms a more dispersed network in the cytoplasm. ×1800.

The treatment of cells with calcium ionophores has dramatic effects on the ER. At the immunofluorescence level this is immediately apparent as a significant dispersal of the ER fluorescence, resulting in it occupying most of the cytoplasm including the peripheral lamellae near the plasma membrane. Upon closer examination it appears that the entire tubular-lamellar configuration of the ER has been transformed into small fragments. Electron microscopy directly confirms that the ER has indeed been
dissociated into small fragments, most of which are circular in outline. Interestingly, however, the outer nuclear membrane, which is continuous with the RER, appears unaffected. Thus we conclude that the effect of the ionophore on the ER is to cause the continuous reticulum to be disrupted into small, relatively uniform fragments and that the disruption of the ER is accompanied by a movement of the fragments towards the cell periphery.

The fragmentation of the ER appears to involve calcium ions since it is induced by both A23187 and ionomycin at concentrations that are known to cause specific effects on intracellular calcium, but not by ionophores that are specific for ions other than calcium. It has not yet been resolved whether the effect of the calcium ionophores is to cause an increased influx of calcium from the medium or whether they cause an unloading of the intracellular pools or both. Whatever the outcome on this issue, the striking effect on ER morphology that results from the perturbation of intracellular calcium suggests that calcium ions are directly or indirectly involved in the stabilization of ER morphology. From this standpoint it is interesting that other agents that are known to cause the disruption of intracellular systems, such as microtubules (colchicine, nocodazole) and microfilaments (cytochalasin b), do not cause the ER to fragment in the same way (see Table 1). Colchicine does cause the ER in fibroblasts to assume a more dispersed state but this does not appear to involve disruption into small fragments. Monensin, which causes

**Fig. 9.** Linear arrays of ER fragments in cells with re-assembling ER. A. An example of a cell in which the ER fragments generated with calcium ionophore and re-incubated in normal medium for 30 min have formed into linear arrays (arrowed) which appear to be incipient forms of the continuous re-formed reticulum shown in Fig. 8. The micrograph is a composite from 3 separate frames. The region marked by the large arrow has been enlarged (B) and areas exhibiting linear arrays of fragments are arrowed. A, ×3300; B, ×10000.
Fig. 10. Calcium-dependence of ER re-assembly from fragments. ER fragmentation was induced with A23187 and the cells then incubated in low-calcium medium (A) or the same medium supplemented with 1 mM-calcium chloride (B). Note that re-assembly is only apparent in the cells incubated with calcium. ×1800.

dilation of the Golgi complex at μM concentrations (Tartakoff & Vassali, 1977), has only a slight effect on the ER under similar conditions. Reciprocally, calcium ionophores do not cause dispersal of the Golgi complex (unpublished observation). These observations indicate that the structure of the ER is particularly sensitive to alterations in intracellular calcium and suggest the possibility that calcium ions may directly participate in the stabilization of the ER.

The dissociation of the ER into fragments by calcium ionophores is clearly a reversible process, since transfer of cells to normal medium causes the fragments to reassemble rapidly into the continuous configuration. Re-assembly is rapid since it can be detected in many cells after as little as 5–10 min and is essentially complete with 30–45 min. The rapidity of the process strongly suggests that the re-formed reticulum is formed directly from the fragments, and indeed it is frequently observed that the
fragments align into linear arrays during the process of re-assembly. Furthermore, the re-formed ER usually exhibits a much more dispersed morphology, consistent with its re-assembly from the dispersed fragments.

The above observations show that it is possible to take the ER of 3T3 fibroblasts through cycles of dissociation and re-assembly by simple treatment with calcium ionophore. This provides a convenient system for the assay of ER assembly in living cells. The process induced with calcium ionophore appears to resemble closely that occurring during the normal mitotic cycle, since in dividing cells there is a similar dissociation and dispersal of the ER into small fragments, which rapidly re-assemble in the daughter cells following division.

The availability of a convenient assay for ER re-assembly in living cells permitted the examination of some of the factors which might affect ER re-assembly in vivo. The most significant observation was that re-assembly is completely dependent on the presence of millimolar levels of calcium ions in the re-assembly medium. This was demonstrated by carrying the process out in medium containing <1 μM-calcium, under which the ER of 3T3 fibroblasts through cycles of dissociation into small fragments, which rapidly re-assemble in the daughter cells following division.

Thus it appears that calcium ions are not only necessary for the maintenance of ER morphology but that they are also necessary for the re-formation of the continuous reticulum. In the latter case there is no uncertainty that the calcium originates from the external medium. Presumably the ionophore treatment leads to a depletion of the intracellular calcium store, which is probably the ER itself (Carafoli, 1987), so that it cannot re-assemble even after removal of the ionophore. All these observations suggest that calcium ions associated with the ER actually participate in the stabilization of the ER, possibly in the way in which they appear to support the structural integrity of the sarcoplasmic reticulum (SR) by interacting with calsequestrin to form a matrix within the lumen of the SR (MacLennan & Holland, 1975). In this context it is worth noting that it has recently been demonstrated that there is a family of abundant low-affinity (i.e. calsequestrin-type) calcium-binding proteins in the ER (Macer & Koch, 1988). Furthermore, calcium ionophores are known specifically to induce the over-expression of the very proteins that can bind calcium in the ER (Resendez et al. 1985; Macer & Koch, 1988). Finally, although in the short term calcium ionophores cause ER dissociation, prolonged incubation (>6 h) of cells in ionophore-containing medium leads to the re-formation of the continuous reticulum. Taken together, the above observations suggest that calcium ions, possibly in association with the calcium-binding reticuloplasmins, contribute directly to the stability of the continuous reticulum.

Recent studies on living cells and in vitro ER assembly systems have suggested a role for microtubules in the assembly of the ER (Terasaki et al. 1986; Dabora & Sheetz, 1987). However, our attempts to demonstrate this directly in our system have not been successful. Since the ER fragments often orient into linear arrays during re-assembly this was an obvious point at which one might expect to observe an association with microtubules. However, no obvious association was observed, although it must be noted that the density of the microtubules is high and this might obscure such interactions to some extent. It is noteworthy that both the above-mentioned studies employed the cyanine-dye DiOC6 as fluorescent marker for ER. In 3T3 cells DiOC6 does not label the same reticular elements as anti-endoplasm and the DiOC6-positive reticulum is not dissociated to the same extent as the endoplasm-positive elements (unpublished observations). Thus it is possible that the involvement of microtubules is particularly significant in the assembly of DiOC6-positive ER, whereas their involvement in the assembly of endoplasm-positive ER, probably rough ER, is less significant. This interesting possibility merits further examination.

Finally, we would like to consider some of the general implications of these studies. The demonstration that the ER can be rapidly dissociated into assembly-competent fragments implies that the organelle can be viewed at least notionally as a system developed from fused subunits. These putative subunits are represented by the uniform fragments generated with calcium ionophore and, in order to emphasize their subunit-like qualities both in the sense of size-uniformity and assembly-competence, we suggest that they be referred to as reticulosomes. Studies are in progress to isolate and characterize the reticulosomes and examine their potential in the assembly of the rough endoplasmic reticulum in vitro.


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