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Subcellular distribution of small GTP binding proteins in pancreas: Identification of small GTP binding proteins in the rough endoplasmic reticulum

(G proteins/cell fractionation/rough microsomes/Golgi/calcium)

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ABSTRACT **Subfractionation of a canine pancreatic homogenate was performed by several differential centrifugation steps, which gave rise to fractions with distinct marker profiles. Specific binding of guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[γ-^{35}S]) was assayed in each fraction. Enrichment of GTP[γ-^{35}S] binding was greatest in the interfacial "smooth" microsomal fraction, expected to contain Golgi and other smooth vesicles. There was also marked enrichment in the rough microsomal fraction. Electron microscopy and marker protein analysis revealed the rough microsomes (RMs) to be highly purified rough endoplasmic reticulum (RER). The distribution of small (low molecular weight) GTP binding proteins was examined by a [α-^{32}P]GTP blot-overlay assay. Several apparent GTP binding proteins of molecular masses 22–25 kDa were detected in various subcellular fractions. In particular, at least two such proteins were found in the Golgi-enriched and RM fractions, suggesting that these small GTP binding proteins were localized to the Golgi and RER. To more precisely localize these proteins to the RER, native RMs and RMs stripped of ribosomes by puromycin/high salt were subjected to isopycnic centrifugation. The total GTP[γ-^{35}S] binding, as well as the small GTP binding proteins detected by the [α-^{32}P]GTP blot overlay, distributed into fractions of high sucrose density, as did the RER marker ribophorin I. Consistent with a RER localization, when the RMs were stripped of ribosomes and subjected to isopycnic centrifugation, the total GTP[γ-^{35}S] binding and the small GTP binding proteins detected in the blot-overlay assay shifted to fractions of lighter sucrose density along with the RER marker.**

There are now a number of examples consistent with the classical paradigm of signal transduction in the plasma membrane, where receptor–effector interactions are mediated by GTP binding proteins (1). Recently, GTP has been implicated in a number of intracellular processes, including the sorting of proteins along the secretory pathway (2) and modulation of intracellular calcium release (3), although the molecular mechanism underlying the GTP effect on these processes has not been clearly defined. It has been suggested that a class of "small" GTP binding proteins (with molecular masses in the 20-kDa to 30-kDa range), distinct from the classical heterotrimeric variety, may be involved in some of these processes (4).

The subcellular distribution of GTP binding in a defined canine pancreatic subcellular fractionation has been examined. Using a blot-overlay assay, the subcellular distribution of "small" GTP binding proteins recognized by this assay has also been examined. In a rough microsomal fraction, which appears to be highly purified rough endoplasmic reticulum (RER) by both marker protein analysis and electron microscopy. Several small GTP binding proteins have been detected. On isopycnic gradients of native and stripped rough microsomes (RMs), these small GTP binding proteins exhibit a behavior indicative of a RER localization.

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MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (≈3000 Ci/mmol; 1 Ci = 37 GBq), guanosine 5′-[γ-[^{35}S]thio]triphosphate (GTP[γ-^{35}S]) (≈1000 Ci/mmol), and ^{125}I-labeled protein A were purchased from DuPont. Antisera were generous gifts from the following investigators: Suresh Tate [Cornell Medical College (canine renal γ-glutamyl transpeptidase)]; David Meyer [University of California–Los Angeles (ribophorin I)]; Rick Wozniak [Rockefeller University (rat liver lamins)].

Subcellular Fractionation and Preparation of RMs. The fractionation was performed exactly as described (5) and a "balance sheet" was kept. For studies of the distribution of GTP binding proteins and cellular markers, an attempt was made to use approximately "equivalent" fractions of the same preparation (Prep 6-20). Thus, the amount of "postmitochondrial" supernatant (see Fig. 2, lane 3) loaded for SDS/PAGE was that which, in the fractionation scheme, would be expected to yield the amount of "postmicrosomal" supernatant, interfacial "smooth" microsomes, cushion fraction, and RMs loaded in lanes 5–8, respectively.

Isopycnic Flotation. RMs, or RMs that had been stripped of ribosomes by 1 mM puromycin/500 mM KOAc/5 mM $MgCl_2$/50 mM tetraethylammonium hydrochloride, pH 7.5, was subjected to flotation through linear 34–65% sucrose gradients as described (5).

Seventeen fractions were collected. The final fraction included the resuspended pellet. Individual fractions were analyzed for the presence of the GTP[γ-^{35}S] binding, small GTP binding proteins recognized by the [α-^{32}P]GTP blot-overlay assay, and ribophorin I.

[α-^{32}P]GTP Blot Overlay Assay. This assay was performed as described by others (6).

GTP[γ-^{35}S] Binding Assay. The filtration assay was performed as described by others (7). Individual filters were dissolved in scintillation fluid and assayed with a Beckman scintillation counter.

Electron Microscopy. RMs were fixed with 2.5% glutaraldehyde in suspension on ice for 15 min, pelleted, and fixed another 30 min on ice, then treated with 1% OsO_4. Samples were then block stained with 2% uranyl acetate, dehydrated, and embedded in Epon. Ultrathin sections were cut and then stained with uranyl acetate and lead citrate.

General. SDS/PAGE, sample preparation for SDS/PAGE, the Western blotting procedure, and the protein assay were as described (5).

Abbreviations: RM, rough microsome; RER, rough endoplasmic reticulum; GTP[γ-^{35}S], guanosine 5′-[γ-[^{35}S]thio]triphosphate.

RESULTS

A detailed analysis of the fractionation scheme depicted in Fig. 1 has been described (5). Briefly, a canine pancreatic homogenate was subfractionated by several differential centrifugation steps. The distribution of GTP[γ-^{35}S] "enriched" binding (total binding/amount of protein) and small GTP binding proteins detected by the blot-overlay assay was examined in approximately equivalent fractions (defined in *Materials and Methods*), which are distinguished by their marker profile (Fig. 2) and sedimentation behavior.

The distribution of the RER was defined by a well-characterized RER marker, ribophorin I. Approximately half the RER fractionated into the 800 × *g* ("nuclear") pellet (Fig. 2*D*, lane 2), consistent with its known association with the nuclear envelope (Fig. 2*C*, lane 2), and the possibility that RER, like plasma membrane (Fig. 2*B*, lane 2), may sediment as large sheets. When the 800 × *g* ("postnuclear" supernatant; Fig. 2*D*, lane 1) was sedimented at 10,000 × *g*, approximately half of the RER was found in the "mitochondrial" pellet (Fig. 2*D*, lane 4), consistent with the described cosedimentation of the RER with mitochondria (8). Further fractionation of the postmitochondrial supernatant (lane 3) over a 1.3 M sucrose cushion gave rise to four distinct fractions: a postmicrosomal supernatant (lane 5, representing cytosol), an interfacial "smooth microsomal" fraction [lane 6, which, based on previous density centrifugation data (9) and morphometric studies (10), is likely to be composed primarily of Golgi and smooth vesicles], the cushion itself (lane 7), and the "rough microsomal" pellet (lane 8).

As is evident in Fig. 2*D*, virtually all the ribophorin I present in the postmicrosomal supernatant (lane 3) distributed into the rough microsomal pellet (lane 8). This pellet was free from contaminating plasma membrane (Fig. 2*B*, lane 8) and nuclei (Fig. 2*C*, lane 8). Electron microscopy of the rough microsomal pellet revealed that >99% of the vesicles seen in cross-section had one or more attached ribosomes (see Fig. 4). Thus, the rough microsomal pellet appeared to be highly purified RER.

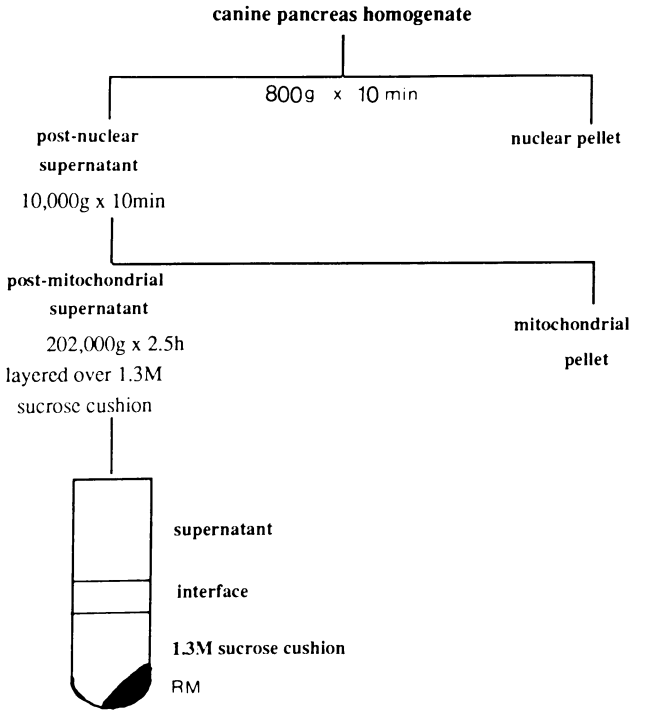

FIG. 1. Subcellular fractionation scheme. A canine pancreatic homogenate was subfractionated according to this scheme. Individual subfractions (equivalent amounts) were subsequently analyzed for the presence of marker proteins and small GTP binding proteins. The RM (postmicrosomal) pellet is shown.

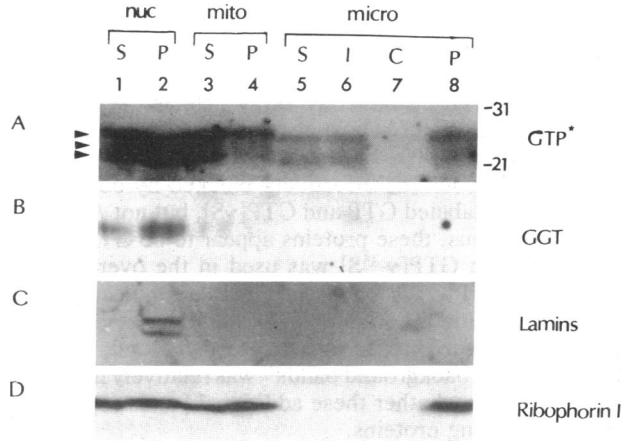

FIG. 2. Distribution of small GTP binding proteins in approximately equivalent subcellular fractions of canine pancreas (Prep 6-20). Lanes: 1, postnuclear supernatant; 2, nuclear pellet; 3, postmitochondrial supernatant; 4, mitochondrial pellet; 5, postmicrosomal supernatant; 6, interfacial smooth microsomes; 7, cushion; 8, RMs. (*A*) [α-^{32}P]GTP blot-overlay assay, with apparent molecular masses indicated to the left in kDa. (*B*) Immunoblot with antisera against glutamyl transpeptidase (GGT, plasma membrane marker). (*C*) Immunoblot with antisera against ribophorin I (endoplasmic reticulum marker). S, supernatant; P, pellet; I, interface; C, cushion.

Despite their distinct marker profiles and sedimentation behavior, substantial GTP[γ-^{35}S] binding was found in virtually all the fractions (not shown). Values for enriched binding (total GTP[γ-^{35}S] binding divided by protein amount) are given in Fig. 3. Clearly, marked enrichment of GTP[γ-^{35}S] binding was found in several fractions: the interfacial smooth microsomes (fraction 6), the cushion (fraction 7), and RMs (fraction 8). The enrichment was greatest in the interfacial smooth microsomal (fraction 6), perhaps reflecting the involvement of GTP along the secretory pathway in Golgi and transport vesicles (4), both of which would be expected to be

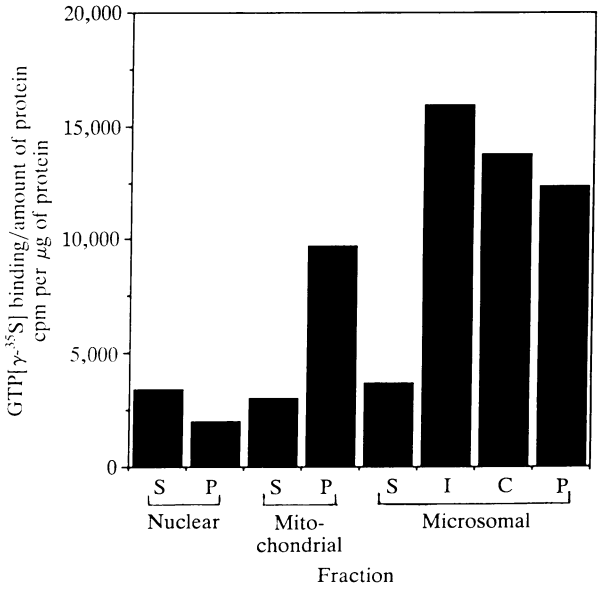

FIG. 3. Enriched binding of GTP[γ-^{35}S] in approximately equivalent pancreatic subfractions. Binding was measured in duplicate by a filtration assay (7). Bars correspond to lanes 1–8 described in Fig. 2 legend. Enriched binding (cpm per μg of protein) in individual subfractions was determined by dividing total binding by amount of protein.

enriched in this fraction. Specific binding was almost as high in RMs (fraction 8), suggesting the possible presence of GTP binding proteins in the RER.

The [α-^{32}P]GTP blot-overlay assay detected several proteins of 22, 23, and 25 kDa in each of the fractions in which substantial total GTP[γ-^{35}S] binding was found. The binding of [α-^{32}P]GTP could be effectively blocked by competition with excess unlabeled GTP and GTP[γS], but not ATP (data not shown). Thus, these proteins appear to be GTP binding proteins. When GTP[γ-^{35}S] was used in the overlay assay instead of [α-^{32}P]GTP, bands of the same molecular mass were detected (data not shown). In addition, several other bands in the 20- to 30-kDa range were also found; however, the number of "background bands" was relatively high; thus, it was not clear whether these additional bands represented true GTP binding proteins.

The subcellular fractionation data indicated that small GTP binding proteins were present in two distinct fractions in which binding was greatly enriched: the smooth microsomal fraction (Fig. 2*A*, lane 6) and the rough microsomal fraction (lane 8). The results of a number of blots show two or three bands of 22, 23, and 25 kDa in each of these fractions. However, in RMs, the 25-kDa band generally gave greater labeling than the smaller molecular mass bands. In addition, small GTP binding proteins were clearly present (as expected) in the nuclear pellet into which most of the plasma membrane distributes (Fig. 2*A*, lane 2) and were found in the cytosolic fraction as well (lane 5).

Although by using biochemical markers (Fig. 2, lane 8) and electron microscopy (Fig. 4) the RMs were shown to be highly purified RER, it remained conceivable that another organelle might unexpectedly cofractionate with the RER in the fractionation scheme. To show that the small GTP binding proteins present in the rough microsomal fraction were indeed in the RER (and not in a membrane that cofractionated with RER), purified RMs were subjected to isopycnic sucrose-density centrifugation. It was found that the GTP[γ-^{35}S] binding (Fig. 5) in RMs and the small GTP binding proteins (Fig. 6*A*) were isodense with the RER marker ribophorin I. The relatively high density at which these proteins were found virtually excludes other membranes (such as Golgi, plasma membrane, smooth endoplasmic reticulum). Moreover, when the RMs were stripped of ribosomes (11) with puromycin/high salt, a treatment that extracts ribosomes, the GTP[γ-^{35}S] binding (Fig. 5) and the small GTP binding proteins (Fig. 6*B*) shifted to lighter densities with the RER marker. Such behavior after treatment with puromycin/high salt would not be predicted for any membrane other than the RER.

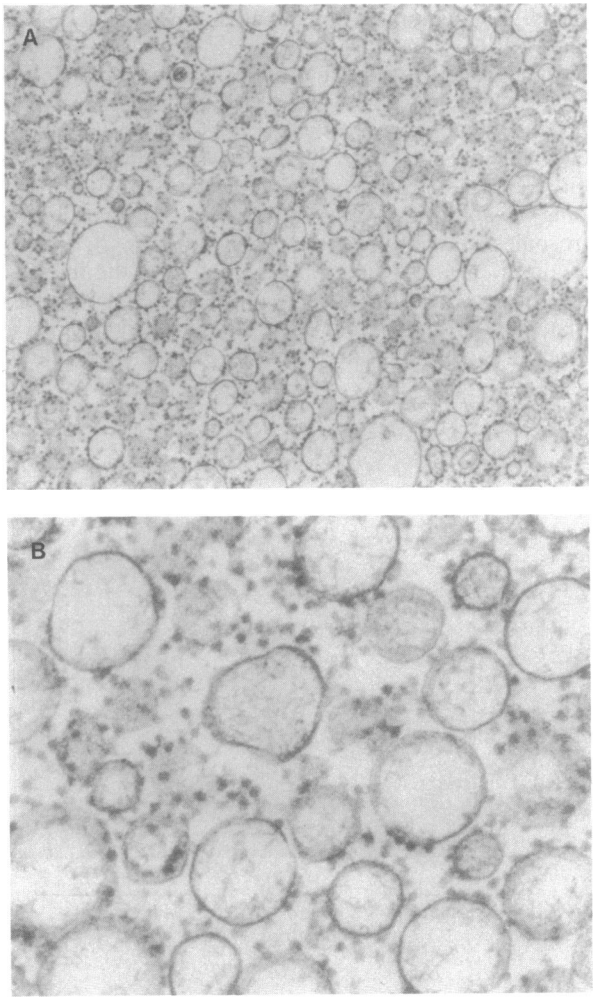

FIG. 4. Electron micrographs of RMs. Standard transmission electron microscopy was performed on the rough microsomal pellet fraction. Note that at least one ribosome is attached to virtually every vesicle. Sections not seen in direct cross-section represent "grazing" sections. (*A*, ×8000; *B*, ×26,400.)

DISCUSSION

A defined fractionation of canine pancreas has been performed and the subcellular distribution of GTP[γ-^{35}S] binding, which would be expected to detect "classical" heterotrimeric G proteins, as well as other GTP binding proteins, was examined. The enriched binding was greatest in the interfacial smooth microsomal fraction (Fig. 3, fraction 6). Based on previous studies (9, 10), this fraction would be expected to contain primarily Golgi, as well as other smooth vesicles, some of which are likely to be involved in transport of proteins between various intracellular compartments (4). Thus, this high enriched binding is consistent with studies suggesting a GTP requirement for protein sorting between various compartments along the secretory pathway (4), probably mediated by transport vesicles.

These binding studies also revealed substantial enriched binding in RMs (Fig. 3, fraction 8). By marker protein analysis (Fig. 2, lane 8) and electron microscopy (Fig. 4), this fraction was shown to consist of highly purified RER. To

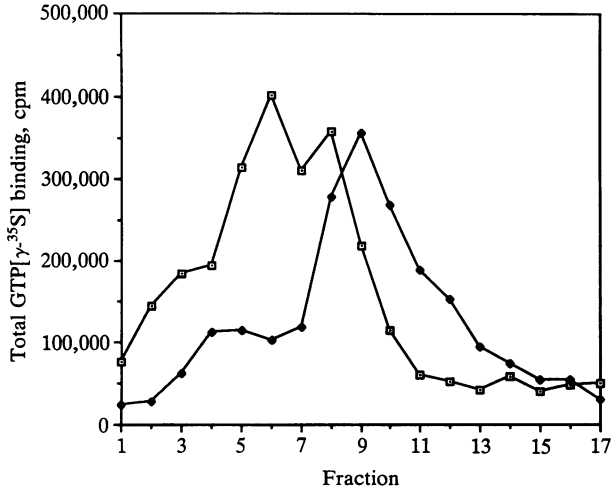

FIG. 5. GTP[γ-^{35}S] binding of fractions after isopycnic centrifugation of native and stripped (puromycin/high salt) RMs over 34–65% linear sucrose gradients. Flotation protocol, stripping procedure, and binding assay are described in text. Fractions 15–17 represent the load zone. The pellet is included in the final fraction. ◆, Native RMs; ⊡, stripped RMs.

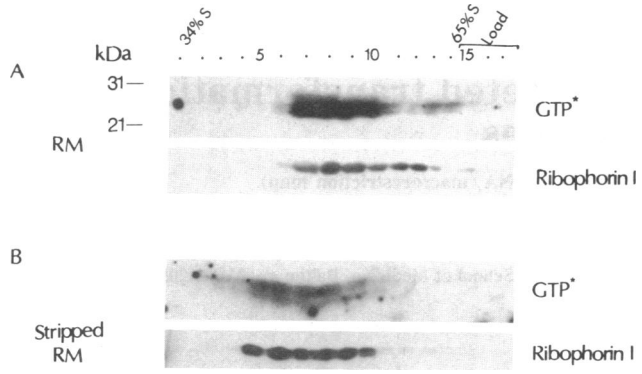

FIG. 6. [α-^{32}P]GTP blot overlay of fractions after isopycnic centrifugation of native and stripped (puromycin/high salt) RMs. Flotation protocol, stripping procedure, and binding assay are described in text. Fractions 15–17 represent the load zone. The pellet is included in the final fraction. (These sucrose gradients, although done the same way as those in Fig. 5, represent a different experiment and are therefore not directly comparable.) (*A*) Native RMs: top subpanel, [α-^{32}P]GTP blot overlay; bottom subpanel, ribophorin I immunoblot. (*B*) Stripped RMs: top subpanel, [α-^{32}P]GTP blot overlay; bottom subpanel, ribophorin I immunoblot; S, sucrose concentration.

further establish that this binding is indeed in RER, the RMs were subjected to isopycnic centrifugation and showed that the binding fractionates at high sucrose densities (Fig. 5). Moreover, when the RMs were stripped of ribosomes with puromycin/high salt, the binding shifted to lighter sucrose densities. Other organelles (potentially contaminating the RMs) would not be expected to behave in this manner.

A search for specific GTP binding proteins using a [α-^{32}P]GTP blot-overlay assay (Fig. 2*A*) was performed. Several GTP binding proteins in the 20- to 25-kDa range were detected in various fractions [including the nuclear pellet, into which most of the plasma membrane distributes (lane 2), and cytosol (lane 5)]. In the Golgi-enriched smooth microsomal fraction (lane 6) and the rough microsomal fraction (lane 8), at least two proteins of 25 and 22 kDa were present. In RMs, these proteins were isodense with the RER marker on isopycnic sucrose gradients (Fig. 6*A*) and shifted to lighter densities with RER marker when the ribosomes were stripped off by puromycin/high salt treatment (Fig. 6*B*). Thus, these proteins appear to be small GTP binding proteins in the RER.

The capacity of the [α-^{32}P]GTP blot-overlay assay to detect all small molecular mass GTP binding proteins, at least in crude fractions, remains unclear. When GTP[γ-^{35}S] was used instead of [α-^{32}P]GTP in the same assay, several additional bands were noted, but their specificity for GTP seemed less clear-cut than those detected with the [α-^{32}P]GTP assay. The GTP binding proteins in the various subfractions, which have distinct marker profiles and sedimentation behavior, could not be distinguished by their migration on standard SDS/PAGE (Fig. 2*A*). It has been suggested that some of these may be involved in shuttling of proteins in the secretory pathway between different cellular compartments (4); thus, the same protein may be found in more than one cellular compartment upon cell fractionation. Nevertheless, given the number of small GTP binding proteins sequenced (4), it is also likely that, even though they are indistinguishable in the blot-overlay assay under standard SDS/PAGE conditions, a number of the proteins found in different fractions are different proteins.

The RER (or a subcompartment of the RER) appears to be a site of inositol triphosphate (IP_3)-sensitive intracellular calcium stores, although this has been debated (12). An IP_3 receptor has been localized to the RER in cerebellar Purkinje cells (13). Consistent with this, in canine pancreas, calcium binding proteins appear to localize to the RER or an associated structure (unpublished observation). The localization to the RER of cAMP-dependent protein kinase (5), which has been shown to phosphorylate the IP_3 receptor and thus modulate calcium release from microsomes (14), suggests physiological significance to this colocalization with proteins involved in calcium storage and release, with the RER presumably serving as an instrument for "cross-talk" between two cellular signaling systems. GTP has been implicated in a different kind of modulation of cellular calcium. It has been suggested that GTP may play a role in determining the distribution of nonmitochondrial calcium stores between IP_3-sensitive and -insensitive pools within the cell (15), possibly mediated by a small GTP binding protein in the RER. As discussed, a role for GTP has also been implicated in movement of translocated polypeptides through the secretory pathway (4), as well as in fusion events between RER vesicles (16). If any of the GTP binding proteins that have been described in the RER are involved in RER to Golgi sorting, they may ultimately localize to transitional elements of the RER.

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