Inducible Dimerization of RET Reveals a Specific AKT Deregulation in Oncogenic Signaling*

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Inducible activating mutations in the RET (rearranged during transfection) proto-oncogene, a receptor tyrosine kinase, are causally associated with the development of multiple endocrine neoplasia type 2A (MEN2A) syndrome. Such oncogenic RET mutations induce its ligand-independent constitutive activation, but whether it spreads identical signaling to ligand-induced signaling is uncertain. To address this question, we designed a cellular model in which RET can be activated either by its natural ligand, or alternatively, by controlled dimerization of the protein that mimics MEN2A dimerization. We have shown that controlled dimerization leaves proximal RET signaling intact but impacts substantially on the tuning of the distal AKT kinase activation (delayed and sustained). In marked contrast, distal activation of ERK remained unaffected. We further demonstrated that specific temporal adjustment of ligand-induced AKT activation is dependent upon a lipid-based cholesterol-sensitive environment, and this control step is bypassed by MEN2A RET mutants. Therefore, these studies revealed that MEN2A mutations propagate previously unappreciated subtle differences in signaling pathways and unravel a role for lipid rafts in the temporal regulation of AKT activation.

The RET4 proto-oncogene is located on chromosome 10q11.2 and encodes a receptor tyrosine kinase with four cadherin-related motifs and a cysteine-rich domain in the extracellular domain (1). It associates with ligand-specific co-receptors known as GFRαs (GDNF, glial-cell-line-derived neurotrophic factor, family receptors α), to form functional receptors for the GFL (GDNF family receptors). In the current view, homodimeric GFL binding induces a GFL2-GFRα2-RET2 complex (2). RET dimerization leads to increased receptor kinase activity and auto-phosphorylation of cytoplasmic tyrosine residues, which serve as docking sites for Src homology 2 (SH2)- and phospho-tyrosine-binding domain-containing proteins, such as Shc or phospholipase Cγ (3). These proteins then recruit additional effector molecules, resulting in the assembly of signaling complexes and the activation of intracellular signaling pathways, including the Ras/extracellular-regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/AKT pathways. GFL-mediated signaling pathways are involved in the development and maintenance of a broad spectrum of neuronal subpopulations (1).

Recently, membrane microdomains, or lipid rafts, have been shown to profoundly influence the functional impact of GDNF-stimulated RET downstream signaling (4, 5). Lipid rafts are suggested to be lateral microdomains in membranes of living cells, enriched in sphingolipids, cholesterol, and specific membrane proteins. They are characterized by higher order and by having a lower buoyant density than bulk plasma membranes (6, 7). Although uncertainties about the precise molecular nature of rafts remain (8–11), compelling evidence indicates that lipid rafts can coalesce into larger and more stable structures where proteins can segregate to perform functions (12, 13). With regard to GDNF-stimulated RET signaling, it has been shown that at steady state, GFRα1, but not RET, is a resident of lipid raft-related detergent-resistant membranes (DRMs). GDNF stimulation was demonstrated to trigger RET recruitment and activation into DRMs. This was strongly correlated with downstream signaling, cell survival, and differentiation (4). Furthermore, Paratxa et al. (5) showed that RET association with DRMs may influence the nature of the intracellular signaling. Together, these studies demonstrate a connection between RET association with DRMs and RET signaling and support a role for lipid rafts in controlling GDNF-stimulated RET signaling.

Mutations in the RET proto-oncogene have been identified as causative for human papillary thyroid carcinoma, multiple endocrine neoplasia (MEN) type 2 A and B, and familial medullary thyroid carcinoma (14). MEN2A is an autosomal dominant cancer syndrome, characterized by medullary thyroid carcinoma, pheochromocytoma, and hyperplasia of the parathyroid gland. MEN2A mutations were identified in the cysteine-rich region, and ~90% of those mutations affect codon 634 (most frequently a cysteine to arginine change) (15). They result in a constitutive activation of RET through formation of covalently linked dimers of the receptor, independent of GFL (16, 17). Dimerization occurs early during the maturation process and results in additional activation of incompletely glycosylated intracellular RET precursors (18). Whether MEN2A signaling is identical to GFL-triggered signaling is difficult to answer because activation of the constitutive MEN2A protein cannot be triggered in resting cells, thus precluding a qualitative and quantitative comparison with the GFL-inducible signaling pathways. Consequently, the current model to explain how MEN2A RET mutants promote tumorigenesis only considers the permanent nature of MEN2A signaling.
To address this issue, we have generated a chimeric RET-Fv protein that can be alternatively activated in the same cell, either with the natural ligand GDNF or with a synthetic bivalent dimerizing ligand (19). The latter mimicked ligand-independent RET dimerization by MEN2A mutants. This induced activation of RET specifically resulted in altered AKT activation but not ERK activation. Furthermore, we demonstrated that a lipid-based cholesterol-sensitive environment regulates the tuning of GDNF-induced AKT activation, suggesting a role of lipid rafts. Finally, we showed that MEN2A mutants escape from this control step. Therefore, these studies revealed complex alterations in oncogenic signaling by MEN2A RET mutants.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents, and DNA Constructs—Phospho-tyrosine (4G10), hemagglutinin (HA), Biotin, and human transferrin receptor antibodies were from Upstate Biotechnology (Lake Placid, NY), Covance Research (Berkeley, CA), BD Transduction Laboratories, and Zymed Laboratories (South San Francisco, CA), respectively. Protein-specific anti-phospho antibodies were from Cell Signaling Technology (Beverly, MA). Anti-RET was described elsewhere (20). Anti-phospho-RETs (Tyr-1015 or Tyr-1062) were generously provided by Dr. Massimo Santoro (21). Lipofectamine 2000, human GDNF, protease inhibitors, monensin, and brefeldin A were from Invitrogen, Promega Corp. (Madison, WI), Roche Diagnostics, BD Biosciences, and Calbiochem, respectively. MTT, Triton X-100, methyl- 

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in which both the mature and the immature forms of RET are activated. Despite the additional activation of the immature form of RET, a comparable increase in tyrosine phosphorylation of cellular proteins was detected after either GDNF or AP stimulation (Fig. 1B, lower panel). It would appear that the addition of the Fv module to the cytoplasmic tail of RET does not obviously alter RET activation and signaling since a similar pattern of tyrosine phosphorylation was observed when cells were transfected with wild type RET instead of RET-Fv and stimulated with GDNF (Fig. 1B). Taken together, these results indicated that homodimerization of RET can lead to its efficient activation without the requirement for GDNF/GFRα1 complex. They also suggested that activation of the intracellular immature 162-kDa Ret protein does not contribute much to the RET-dependent pattern of tyrosine phosphorylation.

GDNF/GFRα1-independent RET Activation Leads to an Altered Cellular Outcome—Next, we investigated whether activation of RET by AP or GDNF can differently affect biological outcomes. RET-Fv/GFRα1-expressing cells were chronically stimulated with either GDNF or AP, and the growth rate was monitored. Neither AP nor GDNF stimulated growth rate during the first 6 days of culture (Fig. 2). After 8 days of culture, both non-stimulated and GDNF-stimulated cells grew in an even monolayer and displayed normal contact inhibition, as shown on representative May-Grunwald-Giemsa staining of cells after 10 days of culture. As a consequence, these cells stopped growing. In contrast, AP-stimulated cells began to overgrow one another, indicating that they had lost the ability to be contact inhibited. These results indicated the existence of specific AP-mediated cellular outcome(s), suggesting that GDNF/GFRα1-independent RET activation leads to alterations of intracellular signaling pathways.

GDNF/GFRα1-independent RET Activation Impacts on AKT Regulation—Next, we tested whether activation of RET by AP or GDNF can translate into different, or differently regulated, RET-mediated signaling pathways. We first monitored the extent of RET autophosphorylation using two other antibodies specific to phosphorylated Y1015RET and Y1062RET. Y1015RET and Y1062RET. Together with the comparable phosphorylation of Y905RET depicted in Fig. 1, these results indicate that AP or GDNF stimulation leads to similar activation of RET tyrosine kinase. It is of note that RET phosphorylation in Rat-1 cells remained high after 2 h of stimulation and that it could last for 6 h before declining (see Fig. 3A). Similar results were obtained with wild type RET instead of RET-Fv (not shown), indicating that the Fv-binding module is not involved in the long lasting phosphorylation of RET in Rat-1 cells. Y1015RET and Y1062RET are binding sites for PLCγ and Shc/Dok4--5/IRS-1/FRS-2, respectively. The bound molecular partners are in turn phosphorylated by RET (3). Fig. 3A shows that both Shc and PLCγ were phosphorylated in a comparable manner following GDNF or AP stimulation and that their kinetics of phosphorylation paralleled those of RET. These results implied that RET activation by AP or GDNF does not influence proximal RET signaling, i.e., autophosphorylation of RET and the resulting phosphorylation of the molecular partners Shc and PLCγ.

We next examined the activation of downstream pathways by looking at ERK and AKT phosphorylation. GDNF induced a transient phosphorylation of ERK and AKT (Fig. 3). Therefore, both Ras/ERK and PI3K/AKT signaling pathways can be attenuated even with the prolonged phosphorylation of RET observed in Rat-1 cells. As compared with GDNF, AP stimulated ERK phosphorylation to a similar amplitude and
duration. However, in marked contrast, the kinetics of AP-stimulated AKT phosphorylation was delayed and sustained for several hours (Fig. 3, A and B). Kinetic alteration of AKT phosphorylation was detected on both serine 473 and threonine 308, which additively activate AKT (28). It should be emphasized that AP does not directly affect AKT activation since no alteration of AKT phosphorylation could be detected following GDNF treatment of cells expressing RET (instead of RET-Fv) in the presence of AP (not shown). These results indicated that GDNF/GFRα1-independent RET activation translates into differently regulated AKT activation, without really affecting ERK activation.

*Neither Signaling from GFRα1 Alone nor from Immature Forms of RET Contributes to AKT Phosphorylation—*Since GDNF has been reported to signal independently of RET via GFRα1 (29, 30), it was important to evaluate whether signaling originating from GFRα1 alone could contribute to AKT and ERK phosphorylation in Rat-1 cells. We thus made use of Rat-1 clones expressing GFRα1 only and have been unable to detect any phosphorylation of AKT and ERK following GDNF stimulation of these clones (Fig. 4A). Next, we evaluated whether AP-activated immature 162-kDa forms of RET-Fv were involved in the differential phosphorylation of AKT. To address this issue, cells were treated with brefeldin A and monensin to abrogate RET maturation and delivery to the plasma membrane (31). The combined use of the two protein maturation blocking drugs was favored because under our experimental conditions, each drug alone was not efficient enough to fully prevent apparition of mature RET proteins (not shown). Cellular treatment for 6 h with the two drugs efficiently blocked the expression of the plasma membrane-localized mature forms of RET while increasing the expression of the endoplasmic reticulum-associated immature 162-kDa form of RET (Fig. 4B). Under these conditions, AP stimulation of cells could still induce phosphorylation of the immature form. Interestingly, phosphorylation of Shc was also detectable, indicating that activated endoplasmic reticulum-associated immature 162-kDa forms of RET can recruit molecular partners. However, these activated forms of RET made no detectable contribution to AKT and ERK activation.
These data, together with the observation that additional activation of immature forms of RET did not modify the tyrosine phosphorylation pattern (Fig. 1B), strongly suggest that immature forms of RET do not contribute to the signaling pathways monitored here.

**GDNF/GFRα1-independent RET Activation Does Not Associate with DRMs**—GDNF/GFRα1-stimulated RET associates with the raft-related DRMs, and this association has been correlated with the control of downstream signaling (4, 5). Thus, to gain an insight into the mechanism(s) responsible for the differential regulation of AKT activation, we asked whether AP stimulation could also induce the association of RET with DRMs. As expected, GDNF stimulation induced an association of mature forms of RET-Fv with DRMs (Fig. 5A). Flotillin and transferrin receptor were used as positive and negative markers, respectively, of DRM-associated proteins. Although RET must associate with GFRα1 to be activated by GDNF, more than 60% of phosphorylated RET-Fv was recovered in soluble fractions (Fig. 5B). This does not seem to reflect a large movement of RET out of DRMs after recruitment and activation since RET remained associated with DRMs for several hours after the beginning of the stimulation in Rat-1 cells (Fig. 5B, and see also Fig. 6A). These results suggested that RET association with DRMs is weaker than GFRα1 association and that it can be partly disrupted by non-ionic detergents. In marked contrast to these results, RET association with DRMs was not evident upon AP stimulation. Similarly, RET proteins carrying a representative MEN2A mutation at Cys-634 (24) were not recovered in the DRM fractions (Fig. 5A). These results clearly demonstrated a difference in the association (or the strength of association) of RET with DRMs between GDNF-dependent and GDNF/GFRα1-independent RET activation.

**RET Association with DRMs Correlates with Specific Regulation of AKT**—The above results raised the possibility that tight regulation of AKT, but not ERK, phosphorylation is linked to DRM association of RET. This hypothesis was tested in two different ways. First, association of proteins with DRMs has been shown to be sensitive to the cholesterol-depleting drug MBC (32). Preincubation of Rat-1 cells for 20 min with 10 mM MBC before stimulation did not affect RET phosphorylation and its total protein level (not shown). However, it significantly delayed the detectable GDNF-induced association of phosphorylated RET with DRMs (Fig. 6A). Quantification by densitometry indicates a 2-h delay in the maximal association of phosphorylated RET with DRMs (see -fold increase). Remarkably, GDNF-stimulated AKT phosphorylation was also delayed, reaching a maximum 2 h after the beginning of the stimulation and declining thereafter. Under these conditions, AP-mediated AKT phosphorylation was barely affected by MBC treatment of the cells, ruling out a massive effect of the drug on cellular membranes (33) and supporting a non-association of RET with DRMs when activated by AP. Interestingly, the AKT phosphorylation level rapidly declined after 2 h of GDNF stimulation in the presence of MBC, whereas it remained high after AP stimulation (Fig. 6A). This indicated that RET association with DRMs correlates not only with a rapid AKT phosphorylation but also with a subsequent efficient AKT dephosphorylation. Altogether, these data underscored a strong correlation between DRM association of RET and a specific regulation of AKT phosphorylation after GDNF stimulation.

Second, we took advantage of the MEN2A mutants that do not associate with DRMs (Fig. 5A). MEN2A mutation at Cys-634 has been shown to activate AKT in Rat-1 fibroblastic cells (24). Because MEN2A mutants can bind to GDNF in the presence of GFRα1 (35), we reasoned that GDNF/GFRα1 stimulation of RET mutants may lead to its associ-
FIGURE 6. Recruitment of RET in DRMs correlates with specific regulation of AKT. A, RET-Fv/GFRα1-expressing cells were pretreated with MBC for 20 min and stimulated with GDNF or AP for the indicated times. Pooled DRMs and soluble fractions (as described in the legend for Fig. 5) were immunoblotted with an anti-phospho-tyrosine antibody to analyze the distribution of phosphorylated RET proteins (α-pY(RET)). Recruitment of phosphorylated RET within DRMs was quantified by densitometry analysis (-fold induction). AKT phosphorylation was revealed with anti-phospho-S473AKT antibody (α-pAKT). B, DRMs from Mα cells stimulated with GDNF for the indicated times were separated as described in the legend for Fig. 5. MEN2A RET mutant proteins distribution within fractions was analyzed by immunoblotting with anti-phospho-tyrosine (α-pTyr) or anti-RET (α-RET). C, decreased AKT phosphorylation was revealed by immunoblotting with anti-phospho-S473AKT, anti-AKT served as loading control. The ratio of the normalized densitometric value of pAKT to total AKT was indicated. D, TUNEL assay in Mα cells stimulated with GDNF for 1 h and subjected to anisomycin (aniso) treatment for 5 h. 4′,6-Diamidino-2-phenylindole (dapi) staining reveals the total number of cells present on the slide and GFP staining only apoptotic cells. The percentage of apoptotic cells is indicated for each condition. A representative experiment out of three is shown.

DISCUSSION

MEN2A mutations of RET tyrosine kinase receptor result in constitutive activation of RET via ligand-independent dimerization. Whether such mutations spread identical signaling to ligand-induced signaling was previously unclear. The present study revealed more complex alterations of MEN2A oncogenic signaling than previously thought and uncovered a critical node linked to a lipid raft environment that specifically controls the temporal activation of AKT. Ligand-independent Dimerization of RET Exposes an Altered Regulation of AKT Activation—Studying MEN2A signaling to unravel alterations of RET signaling pathways is made difficult by the permanent activation of RET mutants. Indeed, MEN2A protein activation cannot be triggered in resting cells, precluding a rigorous comparison with the GFL-inducible signaling pathways. Thus, we reasoned that mimicking MEN2A activation with an inducible homodimerization system would help us understand the potential deregulation of RET signaling pathways involved in oncogenesis. Several pieces of evidence were obtained through these studies indicating that the addition of a binding module to the C terminus of RET did not obviously alter RET activation and function. This RET activation model readily showed that proximal RET signaling, i.e. RET autophosphorylation and phosphorylation of RET substrates such as PLCγ and Shc, is very similar whether the receptor is activated with the natural ligand GDNF or the synthetic dimerizer AP. Therefore, the GDNF/GFRα1 complex was not mandatory for efficient RET activation. This is in line with the studies of Liu et al. (36), who demonstrated a comparable autophosphorylation pattern of wild type

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active RET and a MEN2A RET mutant. However, our model revealed that ligand-independent activation of RET translates into an altered tuning of the PI3K/AKT pathway, whereas the Ras/ERK pathway remained largely untouched. Thus, these data established that ligand-independent dimerization of RET leads to an altered regulation of specific signaling pathways.

A Role for Lipid Rafts in the Specific Control of the Tuning of AKT Activation—Given the wealth of data on the cellular processes regulated by AKT (28), understanding the molecular mechanisms that underlie the deregulation of AKT activation is obviously of great interest. The RET isoform used throughout these studies is Ret9, in which the auto-phosphorylation site tyrosine 1062 is pivotal in the activation of both Ras/ERK and PI3K/AKT pathways (37). In the current view, phosphorylated tyrosine 1062 drives both AKT and ERK activation through the same initial protein complexes, implicating sequentially Shc or FRS2 and Grb2. It is reasonable to assume that the differential regulation of AKT activation occurs downstream of Grb2 recruitment since proximal RET signaling and ERK activation are comparable whether RET is activated in a ligand-dependent or ligand-independent manner. New insight comes from the finding that a lipid raft environment is involved in this differential regulation of AKT. Although lipid rafts remain elusive membrane domains (8, 9), our findings indicated that a lipid-based cholesterol-sensitive environment plays a key role in the temporal regulation of AKT activation. On the one hand, tight regulation of AKT activation by GDNF showed sensitivity to decrease in membrane cholesterol levels. Although cholesterol depletion can potentially influence general properties of the membrane (33), it barely affected AP-mediated AKT activation, supporting a GDNF-specific sensitivity. On the other hand, cell fractionation experiments showed a clear difference in the (strength of) association of RET with DRMs following GDNF or AP stimulation. Although GDNF triggered an association, AP did not. Although the DRM association is a crude criterion to determine raft association (38, 39), it can be interpreted as a reflection of a lipid raft-related biochemical property of RET. Remarkably, this property is subjected to modulation and correlates with specific AKT regulation, providing strong support for a relationship with a biological process.

Our results are in agreement with a previous report showing that disruption of RET-GFRα1 localization to DRMs did not alter GDNF-induced RET phosphorylation (4). Disruption of localization to DRMs was achieved with the use of a transmembrane version of GFRα1 (instead of a glycosylphosphatidylinositol linkage) that does not associate with DRMs. However, these authors found less robust AKT and ERK phosphorylation with no kinetic alteration. This contrasts with the altered kinetics of AKT phosphorylation and minimal effect on ERK phosphorylation we observed with the ligand-independent activation of RET. However, signaling events beyond 60 min after stimulation were not examined and thus cannot be compared with the late AKT activation we monitored (maximal at 120 min). The discrepancy for ERK activation remains unclear. It could reflect dissimilar constraints of the different cellular background used in the two studies, i.e. immortalized fibroblasts as compared with a neuroblastoma cell line.

How could the association of RET with lipid rafts influence AKT regulation? The local environment at rafts may provide enrichment of distinct signaling targets that promote AKT regulation. For instance, the phosphoinositide lipid phosphatidylinositol-4,5-bisphosphate has been reported to accumulate in lipid rafts (40, 41). Phosphorylation of phosphatidylinositol-4,5-bisphosphate by PI3K generates the important second messenger phosphatidylinositol-3,4,5-trisphosphate that is essential to the progress of the PI3K/AKT signaling cascade. Thus, an association with lipid rafts could conceivably increase the means for RET to trigger the PI3K/AKT cascade, resulting in rapid AKT activation. Conversely, potential raft-associated tyrosine or lipid phosphatases could accelerate the termination of AKT phosphorylation. We have examined the potential involvement of phosphatase and tensin homolog lipid phosphatase in the specific regulation of AKT phosphorylation but found no evidence for its recruitment in DRMs (not shown). Lipid rafts have also been implicated as an alternate mechanism to clathrin-dependent endocytosis (34). Therefore, it is possible that RET activated by GDNF does not follow the same intracellular trafficking route, as MEN2A mutations activated RET. Different intracellular trafficking of receptors can have significant roles in controlling the nature of the activation pathway (45). Thus, it is tempting to speculate that a raft-dependent versus a raft-independent internalization process of RET could contribute to the tuning regulation of AKT activation. Ongoing investigations should clarify these issues.

Controlled Dimerization of RET Provides a Useful Model to Study Some Aspects of Oncogenic MEN2A Signaling—Several pieces of evidence indicated that our model of AP-induced dimerization of RET is pertinent to study MEN2A signaling. First, in both cases, RET was activated by homodimerization of the protein in the absence of GFRα1 and GDNF. Second, similar to what we observed in Rat-1 cells transformed by MEN2A RET mutants, chronic activation of RET with AP resulted in loss of contact inhibition. In marked contrast, contact inhibition did not take place under chronic activation of RET with GDNF. Such a difference is not mirrored in the anchorage-independent growth capability of the cells (27), as both types of cellular stimulation led to a comparable number of formed macroscopic colonies in this assay (not shown). However, this operational test of malignancy may not be suitable to provide insights into the questions we addressed, as expression in cultured fibroblasts of other receptor tyrosine kinases, such as the epidermal growth factor receptor (42, 43), can lead to anchorage-independent growth when activated by ligands. Third, the controlled dimerization of RET predicts that MEN2A-induced elevated AKT activity (24) encompasses an altered tuning of its activation. Indeed, this activity appeared deregulated as when MEN2A mutants were forced to associate with DRMs through an interaction with GDNF/GFRα1, AKT phosphorylation was strongly decreased. Such a decrease is of significance because the cells become more sensitive to anisomycin-induced apoptosis. It has been recently reported that a limited proportion of MEN2A RET mutants (7–8%) may constitutively associate with DRMs (44). We may not have detected such an association because of different experimental conditions, yet the cells are still left with a significant proportion of deregulated AKT. Whether the deregulated AKT activation contributes to loss of contact inhibition has been tentatively addressed by treating the cells with the commonly used PI3K inhibitors LY294002 or wortmannin. However, these drugs on their own lead to important Rat-1 cell death after a few days of culture. Thus, this requires further investigation.

A Reappraisal of MEN2A Signaling—Up to now, the model to explain how MEN2A RET mutants promote tumorigenesis has been left with the permanent nature of MEN2A signaling. Our results have brought new insight in this model. A key question in the field has been whether the intracellular activated RET precursor could contribute to the oncogenic potential of MEN 2A proteins. We have provided evidence that the RET precursor is able to phosphorylate the adaptor Shc but unable to trigger further activation of AKT or ERK. Although an indirect effect of the maturating blocking drugs used cannot be excluded, these results are consistent with a previous report suggesting that an activated RET precursor may not contribute to MEN2A-induced tumorigenesis (46). Thus, our data provided support for MEN2A signaling originating pre-
dominantly from the plasma membrane. In addition, our data revealed a complex situation in which MEN2A signaling is not identical to ligand-induced signaling but propagates qualitative differences in the PI3K/AKT signaling pathway.

In conclusion, the present studies revealed that a lipid raft environment specifically controls the tuning of RET-mediated AKT activation, a control step that appears bypassed by MEN2A mutants. The challenges ahead are to pinpoint the molecular mechanisms that underlie the deregulation of AKT activation and to understand their connection with a lipid-based cholesterol-sensitive environment.

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