ULTRASTRUCTURAL ANALYSIS OF HYPHAL TIP CELL GROWTH IN FUNGI: SPITZENKÖRPER, CYTOSKELETON AND ENDOMEMBRANES AFTER FREEZE-SUBSTITUTION

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SUMMARY

The ultrastructure of freeze-substituted tip cells of *Fusarium acuminatum* was analysed by conventional and high-voltage transmission electron microscopy (HVEM). At least 2 morphologically distinct types of Golgi-like endomembrane cisternae were observed, each existing as single, fenestrated sheets and tubular elements that were often very closely associated with mitochondria. From HVEM observations of thick (0.25 and 0.5 μm) sections, the Spitzenkorper appeared to correspond to an apical mass of vesicles. A network of microfilaments was identified among component vesicles of the Spitzenkorper and adjacent to developing septa. Microtubules were oriented primarily parallel to the direction of hyphal growth and were located in all areas of the cytoplasm, including the tip cell apex. Cytoplasmic vesicles were closely associated with these microtubules. From these observations it is suggested that cytoskeletal elements play important roles in localized cell wall formation. The filasome, a previously unreported type of coated vesicle in fungi, might also be involved in wall synthesis.

INTRODUCTION

Growth of filamentous fungi occurs through apical expansion of hyphal tip cells. Localized cell wall synthesis appears to be responsible for this tip growth as in other cells that grow similarly (e.g. pollen tubes, root hairs, algal rhizoids). Hyphal tip growth among ascomycetous and basidiomycetous fungi is associated with an apical body (Spitzenkorper) and may, therefore, represent a unique system for polarized cell expansion. The geometry of this growth process requires an efficient means for (i) specific long-distance intracellular transport of cell wall precursors and enzymes, and (ii) precise control of fusions between the plasma membrane and certain cytoplasmic components. Among fungi it appears likely that both wall precursors (see Grove, 1978) and at least one important participating enzyme (Bartnicki-Garcia, Bracker, Reyes & Ruiz-Herrera, 1978) are particulate. As a working hypothesis, hyphal tip cell growth may be viewed as the result of a highly polarized system of exocytosis.

Among eukaryotic cells the dictysome is a major component of exocytotic machinery. Cells of most fungal taxa lack this typical cytoplasmic constituent, which has presumably been replaced by primarily tubular elements, and consequently the

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cellular basis of fungal growth has remained largely unknown (see Grove, 1978, for review). Contributing to this lack of understanding is the extremely delicate nature of the hyphal tip cell. Conventional chemical fixation destroys or alters much of the ultrastructure of these cells (Howard & Aist, 1979; Hoch & Howard, 1980), and therefore several possible components of the cell wall synthesizing apparatus are poorly characterized or have gone undetected.

Substantial improvement in preservation of fungal ultrastructure is achieved by using an alternative fixation procedure: freeze-substitution (Howard & Aist, 1979). Use of this technique reveals many more cytoplasmic microtubules, for example, as well as ubiquitous endomembrane cisternae. A further modification of this procedure is reported here as well as a 3-dimensional characterization of Golgi-like elements and the tip cell apex. The possible participation of microfilaments in localized exocytosis is suggested as an additional involvement of the cytoskeleton, apart from a role of cytoplasmic microtubules described elsewhere (Howard & Aist, 1980).

**MATERIALS AND METHODS**

Cultures of *Gibberella acuminata* Booth (*Fusarium acuminatum* Ellis & Everhart (ATCC 32965)) were stored and grown on cellulose membranes according to procedures already described (Howard & Aist, 1977, 1979).

Two protocols of specimen preparation for electron microscopy have been used. The details of 'Schedule B' (Howard & Aist, 1979) have been modified as follows. Initial immobilization of cellular components was achieved by quenching in molten fluoroform ('Freon 23' from Matheson Gas Products, E. Rutherford, NJ). Specimens were substituted in anhydrous acetone (−85 °C) for 2 weeks as previously described (Howard & Aist, 1979). The acetone was replaced twice during substitution. Specimens were then exposed to anhydrous chemical fixatives in acetone as follows: 2% glutaraldehyde (GA) plus 2% OsO₄ at −85 °C for 12 h, at −20 °C for 12 h, and then at 0 °C for 45 min; 2 changes of 2% OsO₄ at 0 °C for 45 min each; 2% OsO₄ at +21 °C for 2 h. Specimens were slowly infiltrated with Epon-Araldite as described previously (Howard & Aist, 1979). The plastic was polymerized at +70 °C.

Thin sections were stained according to a modification (Dr Hitoshi Kunoh, Mie University, Tsu-city, Japan, personal communication) of the method reported by Ohkura & Takashio (1970). Aqueous uranyl acetate (2%) was prepared using glass-distilled water, Millipore filtered and mixed, 9:1 (v/v), with Millipore-filtered isobutyl alcohol. The mixture was agitated on a vortex mixer for 30 min and used immediately. Grids were immersed in the stain for 30 min, and were stained subsequently with Reynold’s lead citrate for 10 min. (Uranyl magnesium acetate (7.5%), when used in place of uranyl acetate, also gave excellent staining of thin sections.) These specimens were observed at 80 kV.

For observations using a high-voltage electron microscope (HVEM), 0.25- and 0.50-μm thick sections were stained with 7.5% aqueous uranyl magnesium acetate in 30% (v/v) ethanol for 3 h at 50 °C, and then with Reynold’s lead citrate for 30 min at room temperature. Thick sections were immersed in the uranyl magnesium acetate and lead citrate solutions during staining. The AEI EM-7, HVEM was operated at 1000 kV.

One purpose of this study was to generate control data for subsequent experiments concerning the effects of an anti-microtubule agent (cf. Howard & Aist, 1980). Since this agent was applied in a 0.1% aqueous solution of dimethyl sulphoxide (DMSO), many observations reported here were obtained from 21 hyphal tip cells exposed (as in Howard & Aist, 1980) to a 0.1% DMSO solution. In addition, 16 hyphal tip cells that received no treatment at all were examined. No ultrastructural differences were recognized between these 2 groups of cells.

Since the hexagonal microvesicles (Howard & Aist, 1979) illustrated here closely resembled
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a polyhedral mycovirus, samples of the organism used (ATCC 32965) were assayed for double-stranded RNA (dsRNA), the nucleic acid usually associated with viruses that inhabit fungi (Lemke, 1976). Results of the following procedure were obtained through the courtesy of Dr Peter R. Day, Connecticut Agricultural Experiment Station, New Haven, CT. Mycelia (2-7 g) were scraped from 8 Petri plates of potato dextrose agar medium after 5 days growth at 22 °C. This material was subjected to the assay for dsRNA described elsewhere (Day et al. 1977). A final volume of one quarter of the preparation was electrophoresed on acrylamide gels.

RESULTS

Serial, thick-section, HVEM analysis of tip cells revealed the 3-dimensional organization of the apical and subapical regions of hyphae (Figs. 1-4). When viewing apices at lower magnifications of the HVEM, the apical cluster of vesicles appeared identical to the light-microscopic image of the Spitzenkörper seen in similar living cells with respect to shape, size and location (Figs. 1-3; cf. fig. 1 of Howard & Aist, 1977). This was especially evident when negatives of the apex from serial, median, longitudinal, thick sections were stacked and viewed simultaneously. The ultrastructural equivalent of the Spitzenkörper appeared to include a cluster of 'apical vesicles', a subtending mass of ribosomes, and microvesicles (Figs. 1-3) lying within a meshwork of microfilaments (see below). The microvesicles and filaments were located mostly within a lumen (Fig. 2, cf. Figs. 1 and 3) in the cluster of apical vesicles.

Mitochondria were located immediately basipetal to the Spitzenkörper region and, along with microtubules, were orientated primarily parallel to the long axis of the cell (Figs. 1-3). Mitochondria occupied an area within the central region of the tip cell and were found in close spatial associations with elements of the endomembrane system (Figs. 1-13). These endomembrane elements initially appeared to represent 5 distinct organelles; (1) tubules (Fig. 1); (2) single, fenestrated sheets (Figs. 1-4); (3) elements containing vesicle-like components (Fig. 2); (4) very narrow tubules (Figs. 4, 5); (5) single, fenestrated sheets and tubules of a different electron density and diameter than above (Fig. 4, cf. 2 and 5). However, regardless of differing morphology it became apparent that some of these elements represented different components of the same organelle. In serial sections, direct connexions between different components were evident (e.g. single fenestrated sheets and wider tubules (Figs. 6, 7, arrows)). Some 'tubules' (Fig. 1) were actually sheets that had been cross-sectioned (Figs. 6-9) and sheets of different spatial orientations were sometimes connected as well (Figs. 5-9). Many of these membranous organelles were closely associated with mitochondria (Figs. 1-9). Fenestrated sheets often encircled portions of mitochondria (Fig. 5).

The above observations of smooth endomembranes permitted 2 basically distinct Golgi-like components to be identified. One component consisted of tubular and fenestrated cisternae ca. 35 nm wide. The other component also consisted of tubular and fenestrated cisternae, but were ca. 70 nm wide and usually exhibited a greater electron density than the former.

Vesicles were often associated with the above-described organelles (Figs. 5-10): microvesicles (20-50 nm diameter) with narrower sheets and tubules (Figs. 5-9); apical vesicles (70-90 nm diameter) with wider cisternae (Figs. 2, 6, 10). In addition,
some microvesicles were concentrated within membrane-bound sacks (Fig. 14) which often exhibited a protruberance at one end.

Microvesicles were at times hexagonal in cross-section and were typically coated with electron-dense material in the form of radiating spikes (Fig. 15). These structures are not considered to be mycoviruses since no trace of dsRNA was detected. Other microvesicles were surrounded by a mass of filamentous material (Figs. 16–18). Microvesicles that were surrounded by this mass of filaments are referred to here as 'filasomes' (from fila, plural filum (filament); -some, soma (body)). Filasomes were located in peripheral regions of the cytoplasm of the tip cell (Figs. 1–4; arrows, Fig. 2), and near both complete and incomplete septa (Figs. 18, 19). Young septal walls also had associated with them a filamentous material which was indistinguishable from and often contiguous with that of filasomes (Figs. 18–21). Furthermore, filaments were observed lying parallel with the long axis of the cell adjacent to the plasma membrane (Fig. 22), within the cytoplasm (Fig. 2), and closely associated with microtubules. The filaments associated with filasomes, septa, microtubules and tip cell walls were ca. 6-0 nm in diameter and therefore are referred to as 'microfilaments'.

The network of microfilaments observed in the Spitzenkörper region (Figs. 23, 24) contained numerous apical vesicles and microvesicles. The extent of this network is not known, but the network appeared to be most dense within the lumen of the 'vesicle cluster', between the apical plasma membrane and the subtending mass of ribosomes (Figs. 2, 23). This network of filaments appeared less concentrated than the filamentous component of filasomes.

Cytoplasmic microtubules were a constant feature of all areas of the hyphal tip cell (Figs. 23–27), and were usually orientated parallel with the direction of growth. Microtubules were frequently seen in the Spitzenkörper region (Figs. 23, 24). The one example of microtubule–plasma membrane contiguity at the cell apex is illustrated in Fig. 24. The end of this microtubule appears dilated. In subapical regions of the tip cell, microtubules were observed in various associations: with other microtubules (e.g. V-shaped arrays (Fig. 25) or laterally, end-to-end (Fig. 26)), most frequently with mitochondria (Howard & Aist, 1979) and vesicles (Figs. 2, 25, 26), and with spindle pole bodies (Fig. 27).

Figs. 1–4. Stereo pairs of adjacent, serial, thick (0.25-μm) sections of a hyphal tip (Figs. 1–3) and nearly contiguous subapical region (Fig. 4) of Fusarium acuminatum. The plane of sectioning is parallel to the long axis of the cell. Fig. 2 is median. Mitochondria (m) lie within the central region of the cell and are maintained in a position ca. 1 μm basipetal to the apex. The Spitzenkörper is composed of a mass of apical vesicles, a lumen which contains microvesicles (mv) and a subtending cluster of ribosomes. Filasomes (Fig. 2, unlabelled arrows) are located near the cell wall basipetal to the apex. Five different views of smooth endomembrane cisternae are evident (1–5; see text). Globular bodies (Fig. 3, large arrow) of unknown nature are typical components of near-apical regions (Figs. 1, 3, 4). Microfilaments appear throughout the cytoplasm (Fig. 2, arrowheads). Microtubules, mt; lipid droplet, l; multivesicular body, mvb. × 16,500. Bar, 1 μm. Total tilt, 12°.
For legend see preceding page.
DISCUSSION

The hyphal tip cell is solely responsible for vegetative growth in the filamentous fungi, and it is the apex of this cell type that constitutes the focal point of polarized cell expansion (see Grove, 1978, for review). Phase-contrast microscope images of hyphal apices in 3 classes of fungi (Ascomycetes, Deuteromycetes and Basidiomycetes) are characterized by an ‘apical body’ (Spitzenkörper). From observations of living cells several investigators have concluded that the Spitzenkörper is somehow related to localized cell wall synthesis (Girbardt, 1955, 1957, 1969; Grove & Bracker, 1970; Howard & Aist, 1977). Ultrastructurally, this delicate Spitzenkörper region exhibits a concentration of cytoplasmic vesicles in all species that have been adequately prepared (see Girbardt, 1969; Grove, 1972). However, the ultrastructural components that are responsible for the phase-shift of light which passes through a living hyphal apex has been a source of controversy. Thus far, no one has published the definitive observation: the simultaneous comparison of a light-microscopic image of a living hyphal apex with an electron-microscopic image of the same region in the same cell. Girbardt (1969) and McClure, Park & Robinson (1968) attributed the phase-shift to the apical cluster of vesicles, whereas Grove & Bracker (1970) held that the ultrastructural equivalent of the Spitzenkörper was not this vesicle cluster but rather a smaller zone within the cluster, composed of microvesicles, ‘tubules’ and ribosomes. Results presented here do not agree with the interpretations of Grove & Bracker (1970), despite the opinion that their conventional chemical fixation of the hyphal tip cell is the finest achieved to date. They site a correlation between the taxonomic distribution of the inner zone with species having a Spitzenkörper, and compare light- and electron-microscopic images of different cells. They state that if apical vesicles did constitute the Spitzenkörper, then members of the Oomycetes, which contain an apical accumulation of vesicles, should exhibit this phase-contrast dense body. However, they may have overlooked the apparent differences in vesicle concentration which may be taxonomically correlated with presence or absence of...
a Spitzenkörper (Girbardt, 1969). This vesicle concentration may well confer a different index of refraction (e.g. lipid component of vesicle membranes (Girbardt, 1969) v. surrounding cytoplasm) which is the only possible means by which a phase-shift can occur. A tightly packed mass of apical vesicles would present a greater shift in refractive index than would a loosely packed vesicle mass. Accordingly, one would not expect those fungi which exhibit a loose arrangement of apical vesicles to have a phase-contrast dense region (Spitzenkörper) at their apices (to wit, all but the septate fungi (Grove & Bracker, 1970; McClure et al. 1968)). Furthermore, in the Zygomycetes, a crescent-shaped accumulation of apical vesicles (Grove & Bracker, 1970) might be expected to cause the crescent-shaped region of phase-shift that has been observed by phase-contrast light microscopy in members of this taxon (Grove & Bracker, 1970). In addition, among the septate fungi which exhibit a lumen within the apical vesicle cluster, one might expect that a corresponding region of different contrast would sometimes be visible by phase-contrast light microscopy within the Spitzenkörper, if in fact apical vesicles were the ultrastructural basis for the Spitzenkörper. This observation has been illustrated using *Rhizoctonia solani* (figs. 36, 37 of Grove & Bracker, 1970).

The present work confirms earlier reports (Girbardt, 1957; Grove & Bracker, 1970) of an additional specialized region within the apical cluster of vesicles. Rather than assigning to this specialized region the identity of ‘Spitzenkörper-equivalent’ (Grove & Bracker, 1970), or artifact of fixation (Girbardt, 1969), it is suggested that this region contains the structural means (i.e. microfilaments) by which integrity of the apical vesicle cluster might be controlled. This suggestion provides a possible explanation for the observation that the Spitzenkörper (i.e. a mass composed of hundreds of vesicles) apparently moves as a single unit within the apical dome of growing hyphae (Girbardt, 1955, 1957, 1969, and unpublished observations by this author). Whether or not the filaments in this area are F-actin remains to be determined. However, with regard to this possibility, several other reports seem relevant: the presence of actin-like protein in cells of *Neurospora crassa* (Allen & Sussman, 1978); the inhibition of tip growth and induction of hyphal tip swelling in other Ascomycetes by cytochalasins A or D (Betina, Micekova & Nemec, 1972; Sweigard,
The presence of α-actinin in membranes of secretory vesicles and the possible role of microfilaments in the secretory process (see Girbardt, 1979; Jockusch et al., 1977; Prentki, Chaponnier, Jeanrenaud & Gabbiani, 1979; and references therein). The presence of F-actin within the Spitzenkörper would also provide a possible means for controlling fusion between secretory vesicles and plasma membrane at a specific site.

An accumulation of filamentous material similar to that reported here in the Spitzenkörper region, as a component of filasomes, and along septal walls has been associated with apparent exocytosis in other cell types. Prentki and co-workers identified the components of a 'microfilamentous network' as F-actin in phalloidin-treated hepatocytes (Prentki et al., 1979). They concluded that secretion of triglycerides was inhibited by phalloidin-induced stabilization of F-actin. Heuser & Reese (1976) reported 'various sorts of microfilaments, including a delicate fuzz around the synaptic vesicles' after improving ultrastructural preservation of synapses by using freeze-substitution. More recently, actin-like filaments in close association with the plasma membrane, 'secretory vacuoles' and Golgi apparatus of lactating guinea-pig mammary gland alveolar cells have been found to bind heavy meromyosin (HMM). In addition, these cells contained microvesicles embedded in meshworks of HMM-decorated filaments (Amato & Loizzi, 1979).

From the above discussion it can be seen that a possible role for actin filaments in secretion may be common to a wide variety of eukaryotic cell types. This is an interesting possibility which, if nothing else, provides an impetus for further study of the filaments in hyphal tip cells of *F. acuminatum*, and in other fungi as well (Byers & Goetsch, 1976; Girbardt, 1979; Patton & Marchant, 1978; Hoch & Howard, 1980).

Fig. 16. The filasome consists of a microvesicle core surrounded by a dense mesh of filamentous material. Thin section. × 104 000. Bar, 100 nm.

Fig. 17. A cluster of filasomes in which the core-vesicles (arrows) are associated with filamentous material. υ, apical vesicle. Thin section. × 80 000. Bar, 100 nm.

Fig. 18. Thin section of a forming septum, cut through the septal pore (sp). Note that the electron-dense material which lies against the plasma membrane appears identical to, and is continuous with, the coating material of a filasome (f). × 72 000. Bar, 100 nm.

Fig. 19. Tangential thin section of the septal pore of a newly formed septum. A network of microfilaments extends along the septum and into the cytoplasm. Vesicle, υ; filasome, f. × 30 000. Bar, 500 nm.

Fig. 20. Microfilaments associated with a septum. Numerous microvesicles and a larger, more electron-dense, apical vesicle can be seen along the upper side of this septum. Thin section. × 58 000. Bar, 100 nm.

Fig. 21. Three filasomes can be seen along this portion of a septal wall: the upper filasome appears flattened against the plasma membrane. The core-vesicles of the 2 upper filasomes lie in other thin sections. × 28 000. Bar, 500 nm.

Fig. 22. Microfilaments (arrow) are evident along the plasma membrane (arrowheads) in this thin section. Cell wall, cv. × 50 000. Bar, 500 nm.
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The observations of Amato & Loizzi (1979), and Girbardt (1979), concerning an association between microvesicles and a meshwork of actin-like filaments, provides one possible interpretation of the filamentous component of filasomes. Similar structures have been reported in centric diatoms and were thought to represent a component of the chitin-synthesizing apparatus (Herth & Barthlott, 1979). Since the cell walls of many fungi, including Fusarium, are composed primarily of chitin, filasomes and the diatom structures very likely have the same function. In this regard it should be pointed out that the microvesicular core of filasomes closely resembles the presumed cytoplasmic conveyor of chitin synthetase in fungi—the chitosome (Bartnicki-Garcia et al. 1978). Herth & Barthlott (1979) suggested that the 'fuzz' associated with these particles near the diatom plasma membrane served to fix the synthetase particles in a specialized region. Girbardt (1979) also proposed an entrapment function for the 'microfilamentous septal belt' in Trametes versicolor. A similar suggestion could be made here for the filamentous component of filasomes and the material associated with forming septa in F. acuminatum.

If one were to consider further the composition and function of filasomes, it would be well to examine possible similarities with coated vesicles (Ryser, 1979, and references therein). It is interesting to note that, under certain conditions, isolated coat structures (i.e. clathrin) will form filamentous aggregates (Woodward & Roth, 1978) that appear identical to the filamentous component of filasomes.

As discussed above, the network of microfilaments within the Spitzenkörper might serve as a mechanism for the control of localized secretion (Jockusch et al. 1977). Exocytosis could also be controlled by modulation of the synthesis and intracellular transport of secretory products. With respect to the latter the close association between cytoplasmic vesicles and microtubules in F. acuminatum suggests a transport function for these microtubules. This suggestion is further supported by experimental

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Fig. 23. A median thin section through a Spitzenkörper region reveals numerous microvesicles and apical vesicles (v) lying within a network of microfilaments, between the apical cell wall (cw) and subtending cluster of ribosomes (r). Microtubules (arrows). × 68000. Bar, 100 nm.

Fig. 24. Higher magnification of a region similar to that shown in Fig. 23, but from a different cell. Note the apical vesicles (v), microvesicles and microfilaments (arrows). A microtubule (mt), oriented parallel to the long axis of the cell, terminates in a dilated configuration at the plasma membrane. Plasma membrane (arrowheads). × 113 000. Bar, 100 nm.

Fig. 25. Thin section of a cytoplasmic microtubule array, oriented parallel to the long axis of the cell. Note the vesicles at the region of microtubule convergence. × 33 500. Bar, 500 nm.

Fig. 26. Numerous vesicles contiguous with cytoplasmic microtubules. Thin section. × 43 000. Bar, 500 nm.

Fig. 27. Thin section of a spindle pole body in F. acuminatum located at the narrow end of a pyriform, perhaps migrating, nucleus. Note the electron-dense bar within the spindle pole body, and several associated microtubules. The spindle pole body appears stretched in the direction of presumed migration. Nuclear envelope, ne; nucleolus, nu. × 26 000. Bar, 1 μm.
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evidence (Howard & Aist, 1977, 1980). Again, it seems possible that such a means for control of exocytosis might be shared by eukaryotic cells of widely divergent taxonomy (see Dustin, 1978).

The smooth endomembrane cisternae (GE) of *F. acuminatum* differ from Golgi apparatus of plants and animals (see Morré, 1977), but most probably represent the equivalent organelle. Plant and animal Golgi cisternae typically consist of a central, non-fenestrated region with fenestrated margins, continuous with a system of tubules and secretory vesicles. The GE of *F. acuminatum* also consist of flattened sheet-like elements but do not exhibit a central non-fenestrated region; the flattened GE of *F. acuminatum* are entirely fenestrated. As in other eukaryotic cell types, tubules extend from the margins of fenestrated regions, but in *F. acuminatum* the tubules are often structurally dissimilar from the flattened cisternae. Plant and animal Golgi bodies are frequently associated with endoplasmic reticulum, whereas the GE of this fungus are usually associated with mitochondria. In this respect the GE of this fungus differs from that of most other eukaryotes (Morré, 1977). Dictyosome polarity is typically expressed in terms of progressive differences in membrane thickness and staining properties from one cisterna to the next within a stack. Even though the GE described here do not exist in a stacked configuration, the differences in staining intensity between fenestrated, flattened regions and connecting tubules may represent a certain degree of such functional differentiation. The GE described in this report are presumed to represent a 'Golgi-equivalent' because (i) Ascomycetes, Deuteromycetes and Basidiomycetes lack Golgi bodies (ii) the GE exhibit structural similarities with true Golgi cisternae, and (iii) the GE are apparently involved in production of secretory vesicles. The question of whether or not the latter presumption is correct can only be answered through the application of cytochemical and autoradiographic techniques for the identification and localization of appropriate biochemical markers.

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