Cell Biology of Conidial Anastomosis Tubes in *Neurospora crassa*

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Although hyphal fusion has been well documented in mature colonies of filamentous fungi, it has been little studied during colony establishment. Here we show that specialized hyphae, called conidial anastomosis tubes (CATs), are produced by all types of conidia and by conidial germ tubes of *Neurospora crassa*. The CAT is shown to be a cellular element that is morphologically and physiologically distinct from a germ tube and under separate genetic control. In contrast to germ tubes, CATs are thinner, shorter, lack branches, exhibit determinate growth, and home toward each other. Evidence for an extracellular CAT inducer derived from conidia was obtained because CAT formation was reduced at low conidial concentrations. A cr-1 mutant lacking cyclic AMP (cAMP) produced CATs, indicating that the inducer is not cAMP. Evidence that the transduction of the CAT inducer signal involves a putative transmembrane protein (HAM-2) and the MAK-2 and NRC-1 proteins of a mitogen-activated protein kinase signaling pathway was obtained because ham-2, mak-2, and nrc-1 mutants lacked CATs. Optical tweezers were used in a novel experimental assay to micromanipulate whole conidia and germ tubes to analyze chemotaxis between CATs during homing. Strains of the same and opposite mating type were shown to home toward each other. The cr-1 mutant also underwent normal homing, indicating that cAMP is not the chemotactic inducer. ham-2, mak-2, and nrc-1 macroconidia did not attract CATs of the wild type. Fusion between CATs of opposite mating types was partially inhibited, providing evidence of non-self-recognition prior to fusion. Microtubules and nuclei passed through fused CATs.

Hyphal fusion (anastomosis) is commonly undergone at two stages during the development of the vegetative colony of a filamentous fungus. It occurs initially between spores or spore germ tubes during the early stages of colony establishment and subsequently between hyphae located behind leading hyphae of the peripheral growth zone of the mycelium. Among other proposed roles, vegetative hyphal fusion is believed to facilitate communication and the transport of water and nutrients within the colony by producing a complex interconnected hyphal network (14).

*Neurospora crassa* is proving to be an excellent model system in which to analyze vegetative hyphal fusion (12, 14). Live-cell analysis of hyphal fusion in mature colonies of *N. crassa* demonstrated that specialized fusion hyphae exhibit positive tropisms by growing (homing) toward each other, and their close vicinity to other hyphae can induce the formation of further fusion hyphae (15). Different stages in the homing and fusion process were defined, and this has been summarized by Glass et al. (14). The nature of the diffusible chemoattractant molecules responsible for these tropisms is unknown (15). A number of mutants unable to undergo hyphal fusion have been isolated. Mutants disrupted in the mak-2 and nrc-1 genes encode a mitogen-activated protein (MAP) kinase and a MAP kinase kinase, respectively. These proteins are orthologs of the MAP kinases (STE11 and KSS11/FUS3, respectively) in the pheromone response pathway of budding yeast (25, 31). Increased phosphorylation of MAK-2 was shown to be correlated with the onset of fusion between conidial germ tubes during colony establishment (31). Another mutant unable to anastomose is disrupted in the ham-2 gene, which encodes a putative transmembrane protein of unknown function (42). An ortholog of ham-2 in budding yeast, FAR11, has been shown to be required for pheromone-initiated cell cycle arrest during mating (21). Although the mechanics of hyphal fusion in mature colonies have been well documented for *N. crassa* (15), the cytology of fusion between conidia and conidial germ tubes of this fungus remains largely unstudied.

Köhler (24) described conidia of the plant pathogen *Botrytis allii* as producing two types of hyphae: germ tubes and fusion hyphae. The fusion hyphae were thinner than the germ tubes, were formed after germ tubes, arose either directly from conidia or germ tubes, and grew toward each other. Recently, Roca et al. (35) described similar specialized cellular elements found in the plant pathogen *Colletotrichum lindemuthianum* and termed them conidial anastomosis tubes (CATs). They showed that the CATs in this species were responsible for fusion between conidia in asexual fruit bodies and that nuclei and organelles moved from one conidium to another through fused CATs. A review of the literature revealed that fusion between spore germ tubes is common, and over 20 species were found to produce structures similar to CATs (35, 36). Köhler (24) first showed fusion between germ tubes of *Neurospora* spp.

The initial aim of the present study was to analyze the morphogenetic process of fusion between conidia and conidial germ tubes of *N. crassa* by using confocal live-cell imaging (9, 16) and low-temperature scanning electron microscopy of frozen-hydrated samples (33, 34). We found that the three types of conidia (macroconidia, microconidia, and arthroconidia) produced by *N. crassa* (40) form CATs that are morphologically distinct and different in behavior from germ tubes. The second aim was to determine whether any evidence for an
inducer of CAT formation could be found. Our results were consistent with a CAT inducer being produced by conidia, CAT production being dependent on conidial density, and the inducer not being cyclic AMP (cAMP). The third aim was to investigate the signaling between CATs during CAT homing. For this purpose, a novel optical tweezer (1) micromanipulation assay was developed. In this way, we were able to show that strains of opposite mating type (mat A and mat a) home toward each other and that cAMP was not the chemoattractant involved in homing. The final aim was to determine why fusion mutants disrupted in the mak-2 (31), nrc-1 (25), and ham-2 (41) genes are unable to undergo CAT fusion. We found that this was because each mutant was blocked in CAT induction, suggesting that transduction of the CAT inducer signal involves the HAM-2 transmembrane protein and a MAP kinase signaling pathway.

MATERIALS AND METHODS

Strains and culture conditions. The *N. crassa* strains used are listed in Table 1. All strains were grown and maintained on solid Vogel's agar medium containing 2% (wt/vol) sucrose (8). When the nrc-1 mutant was used, 2% glucose (instead of sucrose) was added to the medium. Solid or liquid Vogel's medium containing 2% (wt/vol) sucrose (8). When the experiments, unless stated otherwise, conidia were used at a concentration of 10^6/ml immediately after the conidia were harvested. Confocal live-cell imaging was performed by using the signal from the lower secondary electron detector. The trap and move germling was only held in the tweezer trap for <15 s. Bright-field imaging was performed over a 3- to 6-h period to follow different stages of CAT formation, homing, and fusion. Confocal laser scanning microscopy was performed by using a Radiance 2100 system equipped with blue diode and argon ion lasers (Bio-Rad Microscience) and mounted on a Nikon TE2000U Eclipse inverted microscope.

| Strain          | Genotype          | Reference or source   |
|-----------------|-------------------|-----------------------|
| FGSC 2489       | 74-OR23-1VA       | FGSCw                 |
| RLM 40-27       | 74-ORS-A          | 31                    |
| FGSC 4200       | ORS-SL6a          | FGSC                  |
| N2282           | a his-3; :Pccg-hH1::sgfp | 10                    |
| N2283           | a his-3; :Pccg-hH1::sgfp | 10                    |
| N2505           | (ridIP4 A a his-3; :Pccg-1-Bml::sgfp) + ridIP4 mat a his-3 | 10 |
| FGSC 4564       | ad-3B cyh awm3    | FGSC                  |
| FGSC 4000       | cr-1 A            | FGSC                  |
| nrc-1           | at-2 aro-9 nrc-1 inv qa-2 a (qa-2) | 25 |
| PB-1            | mak-2 a           | FGSC                  |
| FGSC 9059       | ham-2 A           | FGSC                  |
| FGSC 9060       | ham-2 a           | FGSC                  |

a FGSC, Fungal Genetic Stock Center.

TABLE 1. Different *N. crassa* strains used in this study

RESULTS

CATs are different from germ tubes. Macroconidia produced both germ tubes and CATs (Fig. 1). The germ tubes were wider and tended to avoid each other, while the thinner CATs grew (homed) towards each other and fused (Fig. 1A to C; also see Fig. 2A to E, 4A, 5A and B, 6A to C, and 7A to C). The actual widths of germ tubes and CATs were strain dependent (Table 2). The germ tubes were longer and exhibited indeterminate growth until they developed into vegetative hy-
In contrast, the CATs were short (<15 μm) and exhibited determinate growth, which commonly resulted in fusion with other CATs. In addition, CATs did not undergo branching, while germ tubes did (Fig. 2). Three patterns of fusion were found to occur between conidia and conidial germings: (i) fusion between CATs produced directly from conidia (Fig. 2A), (ii) fusion between germ tubes that narrowed down at their tips (Fig. 2B), and (iii) fusion between CATs produced as subapical branches from germ tubes (Fig. 2C). Sometimes a CAT formed as a branch just behind a germ tube tip (Fig. 2B). We were unable to convincingly show a direct fusion between a single CAT and a conidium. When we examined samples at high resolution with low-temperature scanning electron microscopy, CAT fusion always seemed to involve two CATs, even if one of the CATs was only discernible as a slight bulge on the conidium surface (for an example, see Fig. 4A).

**Different conidial types produce CATs.** *N. crassa* produces three types of conidia: macroconidia, macroconidia, and arthroconidia (40). Four- to five-day-old cultures of the H1-GFP strain N2283 (Table 1) produced 80% macroconidia, 18% macroconidia, and 2% arthroconidia (n = 400). Approximately similar proportions of macroconidia, macroconidia, and arthroconidia were produced by the wild-type strain and the other H1-GFP strains used in this study (Table 1). All three conidial types were found to produce CATs as well as germ tubes, and CATs from one conidial type homed toward and fused both with CATs of the same conidial type and with CATs from either of the other two types of conidia (Fig. 2D and E).
Fusion occurs between purified macroconidia and microconidia. The conidia were separated into the following two size classes in the H1-GFP strain N2283 by FACS: <4 μm long (predominately microconidia) and 7 to 12 μm long (predominately macroconidia) (Fig. 3A and B). The majority of the microconidia were uninucleate, while the macroconidia possessed 2 to 10 nuclei. The germination and fusion of these samples were then quantified (Fig. 3C). Germination of the purified macroconidia was initiated at ~2 h and reached 98% after 5 h. Macroconidia maintained at 4°C during the FACS analysis germinated faster when the temperature was increased to 25°C than the slower rate of germination of macroconidia maintained continuously at 25°C (data not shown). Fusion between macroconidia and macroconidial germings started after 4 h and reached a maximum of 58% after 7 h (fusion was quantified as the percentage of conidia and conidial germings involved in fusion). Macroconidial germination was initiated after 3 h and reached a maximum of 66% after 8 h. In the remaining 34% of microconidia, the nuclei broke down and the cells died (this was monitored in the H1-GFP strain N2283) (Table 1). Fusion between macroconidia and microconidial germings began after 6 h and had reached 50% after 12 h (Fig. 3C).

An extracellular CAT inducer, which is not cAMP, is produced by macroconidia. The extent of CAT formation was dependent on cell (i.e., conidium) density: CAT formation was common at high density (10^6 macroconidia per ml) and reduced at low conidial concentrations (~10^5 conidia per ml). Solitary germinated macroconidia in these latter preparations rarely produced CATs (Fig. 2F). This suggested that an extracellular inducer was responsible for initiating CAT formation once the macroconidia (which produced the inducer) were above a threshold concentration. We tested whether extracellular cAMP may be the CAT inducer by using a cr-1 mutant (FGSC 4800) which is defective in adenylate cyclase (32, 41) and lacks CAMP (K. Borkovich, personal communication). This strain formed CATs (Fig. 4B; see also Fig. 6B), showing that cAMP was not the CAT inducer.

Signaling between CATs can be demonstrated by using optical tweezers. Our observations were consistent with CATs homing toward each other as a result of the production of a diffusible chemoattractant molecule from their tips. To demonstrate this attraction experimentally, we developed a novel optical tweezer homing assay. Using this assay, we were able to trap an individual conidium or germling and move it relative to another adjacent conidium or germling. We found that it was possible to move a germling which possessed a CAT relative to another germling with a CAT and toward which it seemed to be homing, and then the growth of the two CATs was reori-
entated so that they grew back toward each other (Fig. 4A). The best results were obtained when the CATs were initially 2 to 5 μm apart and then were moved to a distance less than 10 μm apart. Using the optical tweezer assay, homing was consistently observed between CATs of mat A conidia and conidial germlings (n = 20) and between CATs of mat a conidia and conidial germlings (n = 25) (Table 3).

MAP kinase signaling and the HAM-2 protein are involved in CAT induction. mak-2, nrc-1, and ham-2 mutants, which have been previously described as defective in undergoing mycelial hyphal fusion (31, 42), were analyzed. Low-temperature scanning electron microscopy revealed that none of these mutants formed CATs (Fig. 5). When macroconidia of these mutant strains were combined with the wild-type strain of the

FIG. 3. Microconidial and macroconidial germination and fusion after purification by FACS. (A) Sample of predominately microconidia which were <4 μm in length. (B) Sample of predominately macroconidia that were 7 to 12 μm in length. Bar, 25 μm. (C) Time courses of germination and fusion of microconidia and macroconidia purified by FACS.

FIG. 4. Optical tweezer homing assay experiments. (A) Control experiment. At 0 min, CATs (c1 and c2) formed from the thicker tips of germ tubes (g1 and g2) of two macroconidia (a and b) are homing toward each other. At 3 min, the two germlings have been moved apart with the optical tweezers. At 42 min, the CAT tips are reorientating their growth back toward each other. At 90 min, the CAT tips have adhered and fused with each other. (B) CATs (c1 and c2) formed directly from conidia (a and b) of a cr-1 strain lacking cAMP home toward each other. At 0 min, the CATs are homing toward each other. At 7 min, the two CATs have been moved apart with optical tweezers. At 13 min, the CAT tips have reoriented their growth back toward each other. At 43 min, the CATs have adhered and fused. Bars, 10 μm.
same mating type (mat a) labeled with H1-GFP, fusion was never observed between any of the mutants and the wild type (data not shown).

**Mutants defective in MAK-2, NRC-1, or HAM-2 proteins do not produce CAT chemoattractant.** Using the optical tweezers in a different way than described above, individual CATs of the mat a H1-GFP strain were manipulated into a tangential position within 10 μm of individual conidia of mak-2, nrc-1, or ham-2 strains of mat a mating type. Whether the CATs in each case reoriented and homed toward the mutant conidium (or not) was determined. In every case (n = 5 for each H1-GFP mutant combination), no CAT reorientation was observed (data not shown), indicating that the mutant conidia (which lack CATs) did not produce a chemoattractant capable of reorientating a CAT of the H1-GFP strain. In addition, when mixed together, CATs of the H1-GFP strain did not induce the formation of CATs from macroconidia or germ tubes of the mutant strains.

**cAMP is not required for chemoattraction.** Sequencing of the N. crassa genome indicated the existence of three putative cAMP receptors, and thus, it was hypothesized that cAMP may be playing a role as an extracellular chemoattractant in this fungus (6, 11). To test this hypothesis, we used the optical tweezer assay to determine whether the CATs of a cAMP-deficient mutant (cr-1) were attracted toward each other or

| Mating type combination | Homing result (n) | % Fusion |
|-------------------------|-------------------|---------|
| mat A plus mat A        | + (20)            | 38      |
| mat a plus mat a        | + (25)            | 42      |
| mat A plus mat a        | + (12)            | 22      |

* mat A strain, FGSC 2489; mat a strain, N2283 (expressing H1-GFP).

Homing was determined by using the optical tweezer micromanipulation assay.

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![Figure 5](image-url)  
**FIG. 5.** MAP kinase mutants defective in CAT formation. (A) Wild-type strain with CATs (c); (B) cr-1 mutant lacking cAMP produces CATs; (C) Δmak-2 mutant lacking CATs; (D) nrc-1 mutant lacking CATs; (E) ham-2 mutant lacking CATs. Images were obtained by low-temperature scanning electron microscopy. Bar, 5 μm.
not. Homing between CATs of cr-1 was similar to that of the control (cf. Fig. 4B and 4A), indicating that cAMP is not required for CAT homing. Furthermore, we found that this cr-1 mutant undergoes fusion in the center of mature colonies (data not shown).

**Nuclei and microtubules pass through fused CATs.** Before fusion, nuclei exhibited movement back and forth along the CAT, in and out of the germinated spore, and often close to the apical pole of its homing CAT tip (Fig. 6A). After fusion, nuclei passed through the fused CATs (Fig. 6B and C). Prior to fusion, the microtubules were longitudinally organized along the length of a CAT growing toward another CAT (Fig. 7A). After fusion, microtubules extended through the fused CATs from one conidial germling into the other conidial germling and vice versa and, as a result, became intermixed (Fig. 7B). Although germ tubes most frequently emerged from conidia, sometimes a germ tube grew out as a branch from fused CATs (Fig. 7C). Commonly, multiple fusions occurred between germlings and interconnected many germlings (Fig. 1). Very occasionally, three CATs fused (not shown).

**CATs of opposite mating type are attracted to each other, but fusion was partially inhibited.** It is well established that non-self-fusion between hyphae of opposite mating type (mat A and mat a) typically leads to heterokaryon incompatibility and the death of the fusion cell (13). However, what is not known is whether prefusion recognition occurs during the interaction of vegetative cells of opposite mating type. We addressed this question by analyzing the interactions between germinated conidia of strains of opposite mating types (mat A and mat a) that were mixed together. To identify each of the strains in the mixture, a mat a strain (N2283) with H1-GFP targeted to its nuclei was used (Fig. 6C). The optical tweezer homing assay demonstrated that CATs of opposite mating type consistently homed toward and made contact with each other (n = 12) (Table 3). Without using optical tweezers, fusions between mat A plus mat A, mat a plus mat a, and mat A plus mat a were compared. Fusion was indicated by cytoplasmic continuity being achieved by the two interacting CATs. We found that the percentage of fusion between germlings of different mating types was approximately half (22%) of that between germlings of the same mating type (38 to 42%). This indicated that there is some inhibition of fusion and, thus, non-self-recognition between opposite mating types that oc-

FIG. 6. Nuclear migration and CAT fusion between macroconidia. (A) Homing CATs between macroconidia of a mat a strain. Note that a nucleus has migrated to a region just behind the tip in the lower CAT. Nuclei are labeled with H1-GFP (green); membranes are labeled with FM4-64 (red); cell walls are labeled with calcofluor white (blue). The image was obtained by confocal microscopy. Bar, 10 μm. (B) Fused CATs (f) between macroconidia of a mat a strain. Note that a nucleus is migrating through the fused CATs. Staining is as described for panel A; the image was obtained by confocal microscopy. Bar, 10 μm. (C) Fused CATs (f) between macroconidia: one (labeled with nuclear-targeted H1-GFP) is of a mat a strain and the other is of a mat A strain. Nuclei are fluorescing green. This is a merged confocal image and a transmitted bright-field image. Bar, 5 μm.

FIG. 7. (A) Microtubules (labeled with β-tubulin–GFP) longitudinally organized and extending towards CAT tips of macroconidia which are homing toward each other. (B) After fusion, microtubules (labeled with β-tubulin–GFP) have extended through the fused CATs (f) from one macroconidium (c) bearing a germ tube (g) into the other and vice versa and, as a result, have become intermixed. (C) Germ tube (g) which is growing out of fused CATs. Images were obtained by confocal microscopy. Bar, 5 μm.
curs before or at contact and which can, in some instances, inhibit successful fusion.

**DISCUSSION**

We have shown that anastomoses between conidia and conidial germlings of *N. crassa* involve the functioning of a specialized hypha, the CAT. Our detailed characterization of the CAT in this species has demonstrated that it is morphologically and physiologically distinct from a germ tube and the CAT in this species has demonstrated that it is morphologically different from a germ tube because three mutants (*mak-2, nrc-1, and ham-2*) that formed germ tubes did not produce CATs. CATs were also morphologically different from many of the fusion hyphae located behind the leader hyphae of the peripheral growth zone of the mycelium (15). In contrast to CATs, mycellal fusion hyphae are usually wider (typically, 3 to 8 μm in width) and often dichotomously branched. It is not known whether the short, unbranched fusion hyphae (or pegs), which are also common in this region of the mycelium (15), are different from CATs arising from germ tubes.

**Unique characteristics of CATs.** CATs are produced by macroconidia, microconidia, and arthroconidia. They are morphologically different from germ tubes: they are thinner and shorter and do not undergo branching. Physiologically, CATs are different from germ tubes because, unlike germ tubes, they only grow a short distance (i.e., exhibit determinate growth) and home toward each other (germ tubes tend to avoid each other). Finally, CATs are under different genetic control from germ tubes because three mutants (*mak-2, nrc-1, and ham-2*) that formed germ tubes did not produce CATs. CATs were also morphologically different from many of the fusion hyphae located behind the leader hyphae of the peripheral growth zone of the mycelium (15). In contrast to CATs, mycellal fusion hyphae are usually wider (typically, 3 to 8 μm in width) and often dichotomously branched. It is not known whether the short, unbranched fusion hyphae (or pegs), which are also common in this region of the mycelium (15), are different from CATs arising from germ tubes.

**Signaling involved in CAT induction.** We have provided evidence that conidia produce an extracellular CAT inducer because CAT formation is dependent on macroconidial concentration (reducing the macroconidial concentration resulted in the production of fewer CATs, and solitary germinated macroconidia in these preparations rarely produce CATs). Being cell density dependent, CAT induction thus appears to be a form of quorum-sensing behavior (i.e., involving a mechanism in which cells monitor their population density by releasing signaling molecules into the environment). Quorum sensing is a well known phenomenon in bacteria (29) and has recently been shown to regulate cell morphogenesis in the dimorphic fungi *Candida albicans* (7, 17, 26, 38) and *Cerato
cystis ulmi* (18). The CAT inducer was shown not to be cAMP because a *cr-1* mutant which is unable to synthesize cAMP formed CATs.

Evidence was obtained which is consistent with the transduction of the CAT inducer signal involving a putative transmembrane protein (*HAM-2*) and the *MAK-2* (a MAP kinase)- and *NRC-1* (a MAP kinase kinase kinase)-based MAP kinase signaling pathway because mutations in the genes encoding these proteins did not form CATs. Previously, it had been found that mutants disrupted in the *mak-2, nrc-1,* and *ham-2* genes do not undergo hyphal fusion in subperipheral regions of mature colonies (31, 42) and during colony establishment (42), although in these studies, the stage in the fusion process which was blocked was not determined. MAP kinase signaling has also been shown to be involved in the induction of polarized cell growth (shmoo formation) in budding yeast (28) and in the induction of dikaryotic infection hyphae in *Ustilago maydis* (30). However, unlike CAT induction in *Neurospora* which is a response to extracellular signals from cells of the same genotype, the responses in budding yeast and *Ustilago* are due to non-self-interactions involving peptide pheromones which are mating type specific (5, 27).

**Signaling involved in CAT homing.** A novel assay to analyze CAT homing, involving the use of optical tweezer micromanipulation, was developed. Using this technique, the homing responses of CATs originating from different conidia and conidial germlings could be assessed. Furthermore, it was shown that the reactions between CATs of macroconidia of different strains could be easily analyzed if one of the strains was labeled with GFP. The optical tweezer assay provided clear evidence for the existence of as yet unknown diffusible chemotropic signals being involved in the homing response of CATs. Moreover, the results indicated that these chemoattractants were sensed in the region of CAT tips because the latter grew toward each other.

For the slime mold *Dictyostelium discoideum,* it is well established that extracellular cAMP acts as a chemoattractant involved in amoebal aggregation (2). It is also known that cAMP accumulates in the extracellular medium of wild-type *Neurospora* cultures (19, 37). With the discovery that *Neuro-
spora* possesses three G-protein-coupled receptor-like genes, with the proteins encoded being most similar to slime mold cAMP receptors, the possibility that cAMP may serve a similar chemoattractant role in filamentous fungi was suggested (6, 11). However, we found that a *cr-1* mutant lacking cAMP underwent CAT homing. cAMP, therefore, has no apparent role as a CAT chemoattractant in *N. crassa.* Köhler (24) re-
ported that homing of fusion hyphae and CATs is strongly species specific, with a few exceptions where the chemoattrac-
tion was weak. This suggests that the chemoattractant is a molecule which can provide more specificity than cAMP (e.g., a peptide).

Peptide pheromones that orientate hyphal or cell growth toward either other hyphae or other cells are well documented in the sexual phase of *Saccharomyces* (27), *Ustilago* (39), and *Neurospora* (3, 4, 22, 23). However, these chemotropic responses are between different cell types of different genotypes which each produce a cell-specific pheromone. As indicated above, the present study has been primarily concerned with anastomoses between CATs of the same genotype.

Our study of CAT cell biology has provided a new perspec-
tive of a virtually unexplored aspect of the basic biology of *N. crassa.* It also offers an excellent experimental system in which to analyze the mechanistic basis of the induction, homing, and fusion of CATs, particularly in relation to cell signaling.

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