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BRIEF REPORT

Overexpression of *YPT6* restores invasive filamentous growth and secretory vesicle clustering in a *Candida albicans arl1* mutant

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ABSTRACT

Virulence of the human fungal pathogen *Candida albicans* depends on the switch from budding to filamentous growth. Deletion of the Arf GTPase Arl1 results in hyphae that are shorter as well as reduced virulence. How Arl1 is regulated during hyphal growth, a process characteristic of filamentous fungi, yet absent in *S. cerevisiae*, is unknown. Here, we investigated the importance of the Rab6 homolog, Ypt6, in Arl1-dependent hyphal growth and determined that *YPT6* overexpression specifically rescued the hyphal growth defect of an *arl1* mutant, but not the converse. Furthermore, we show that deletion of *ARL1* results in an alteration of the distribution of the Rab8 homolog, Sec4, in hyphal cells and that this defect is restored upon *YPT6* overexpression.

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Arf; hyphal growth; secretion; Rab; yeast morphogenesis

Introduction

Cell shape changes are critical for a range of biological processes, such as neuronal development, and cell shape abnormalities are characteristic of cancer cells. Morphological changes are also crucial for the virulence of a range of plant and human fungal pathogens. *Candida albicans* is a major fungal pathogen of humans that accounts for ~10% of hospital-acquired bloodstream infections, with mortality rate exceeding 30%. The success of *C. albicans* as a pathogen is associated with its ability to switch between different morphological states.[1–3] These dramatic cell shape changes require cytoskeleton reorganization and sustained membrane traffic, resulting, in particular, in the secretion of hydrolytic enzymes, critical for pathogenicity.[4–6] During filamentous growth, *C. albicans* secretory vesicles are clustered at a structure called the Spitzenkörper at the tip of the filament,[7] the Golgi apparatus redistributes to the apex region[8,9] and endocytosis sites form a collar below the hyphal tip.[8,10] Trafficking to the plasma membrane is mediated by vesicular transport regulated by small GTPases of the Arf (ADP-ribosylation factor) and Rab (Ras-related in brain) families.[11–16]

C. albicans has 5 Arf/Arl homologs compared to 26 in Human. *C. albicans* is a diploid yeast, thought to have diverged from *Saccharomyces cerevisiae* approximately 800 millions years ago.[17] We recently showed that of these 5 Arf/Arl proteins, only Arf2 is essential for viability and antifungal drug sensitivity.[18] While both Arf2 and Arl1 are required for hyphal growth and virulence, Arl1 is additionally critical for restricting hyphal growth to a single site, likely *via* regulation of protein secretion. Arl1 is involved in multiple cellular processes both in mammalian and yeast cells.[19] How is Arl1 regulated during hyphal growth, a process characteristic of filamentous fungi, is at present unknown. Several studies have shed light on the crosstalk between Arf and Rab proteins; in budding yeast, Arl1 was shown to genetically interact with the Human Rab6 homolog, Ypt6.[20–22] Recently, a role of Arl1 and Ypt6 in *S. cerevisiae* autophagy was also reported.[23–25] Here, we investigated the importance of Ypt6 in *C. albicans* Arl1-dependent filamentous growth.

Results and discussion

We have shown that an *arl1/arl1* deletion mutant was dramatically reduced in invasive growth in response to fetal calf serum (FCS) or the carbon source-poor Spider medium.[18] Fig. 1A and 1B show that the *arl1/arl1* defect was partially rescued by over-expression of *YPT6*, on both media. To examine if GTP-GDP cycling of Ypt6 was critical for such a rescue, we generated constitutive active and constitutive negative mutants of Ypt6. Fig. 1C shows that, compared to over-expression of wild-type Ypt6, over-expression of the constitutive negative form of Ypt6, Ypt6[T25N], did not rescue the defect while

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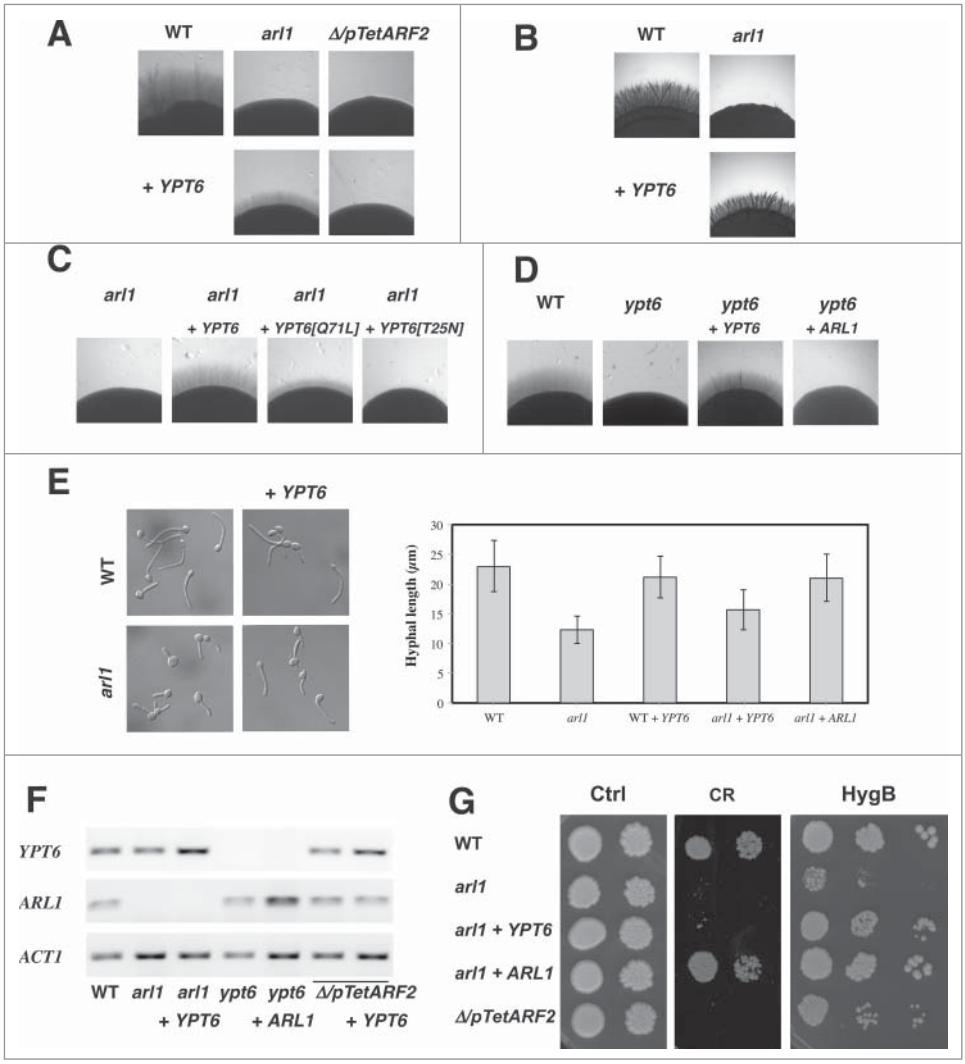

Figure 1. Overexpression of *YPT6* rescues the hyphal invasive growth defect in an *arl1* deletion mutant. (A-D) Overexpression of *YPT6* specifically rescues invasive growth in *arl1/arl1* cells. Indicated strains were grown on agar-containing YEPD with 50% FCS (A, C, D) or on Spider media (B) and images were taken after 5–6 days. Similar results were observed in 2 independent experiments. (C) Rescue of invasive growth in *arl1/arl1* cells depends on Ypt6 activity. Indicated strains were grown on agar-containing YEPD with FCS as in A. (D) Overexpression of *ARL1* does not rescue invasive growth in *ypt6/ypt6* cells. Indicated strains were grown on agar-containing YEPD with FCS as in A. (E) Overexpression of *YPT6* partially rescues *arl1/arl1* hyphal length defect. Cells from the indicated strains were incubated with FCS for 90 min and the graph shows the average hyphal length (mean of 200–400 cells each strain, from 3 experiments); error bars indicate SD. Student *t* test was used to calculate the p values: *arl1* + *ARL1 vs arl1*: 0.0028 and *arl1* + *YPT6 vs arl1*: 0.0042. (F) *YPT6* and *ARL1* transcripts in the overexpression mutants. mRNA and cDNA were prepared from the indicated strains and the transcripts were quantified by RT-PCR; actin (*ACT1*) was used for normalization. (G) Overexpression of *YPT6* does not rescue cell wall defects in *arl1/arl1* cells. Serial dilutions of the indicated strains were spotted on YEPD media (Ctrl) containing 400 μg/ml Congo red (CR) or 1 mg/ml hygromycin B (HygB). Images were taken after 2 days.

over-expression of the constitutive active form, Ypt6 [Q71L], rescued the defect to an intermediate level, suggesting that GTP-GDP cycling of Ypt6 is indeed critical. Alternatively, it is possible that the reduced efficiency of Ypt6[Q71L], compared to Ypt6, to restore invasive growth is due to sub-optimal activation by a GEF.[26] We next investigated the importance of *YPT6* for invasive growth and Fig. 1D shows that *YPT6* is also critical, as a *ypt6/ypt6* deletion mutant was dramatically reduced in invasive growth. This defect in invasive growth of the *ypt6/ypt6* mutant was complemented by over-expression of *YPT6*, but not over-expression of *ARL1*. The deletion and over-expressing mutants were verified by RT-PCR (Fig. 1F and 2G).

Previously, we also observed that the *arl1/arl1* mutant had reduced hyphal growth in response to FCS in liquid media, with filaments ~2-fold shorter compared to that of the wild-type.[18] Fig. 1E shows that *arl1/arl1* cells

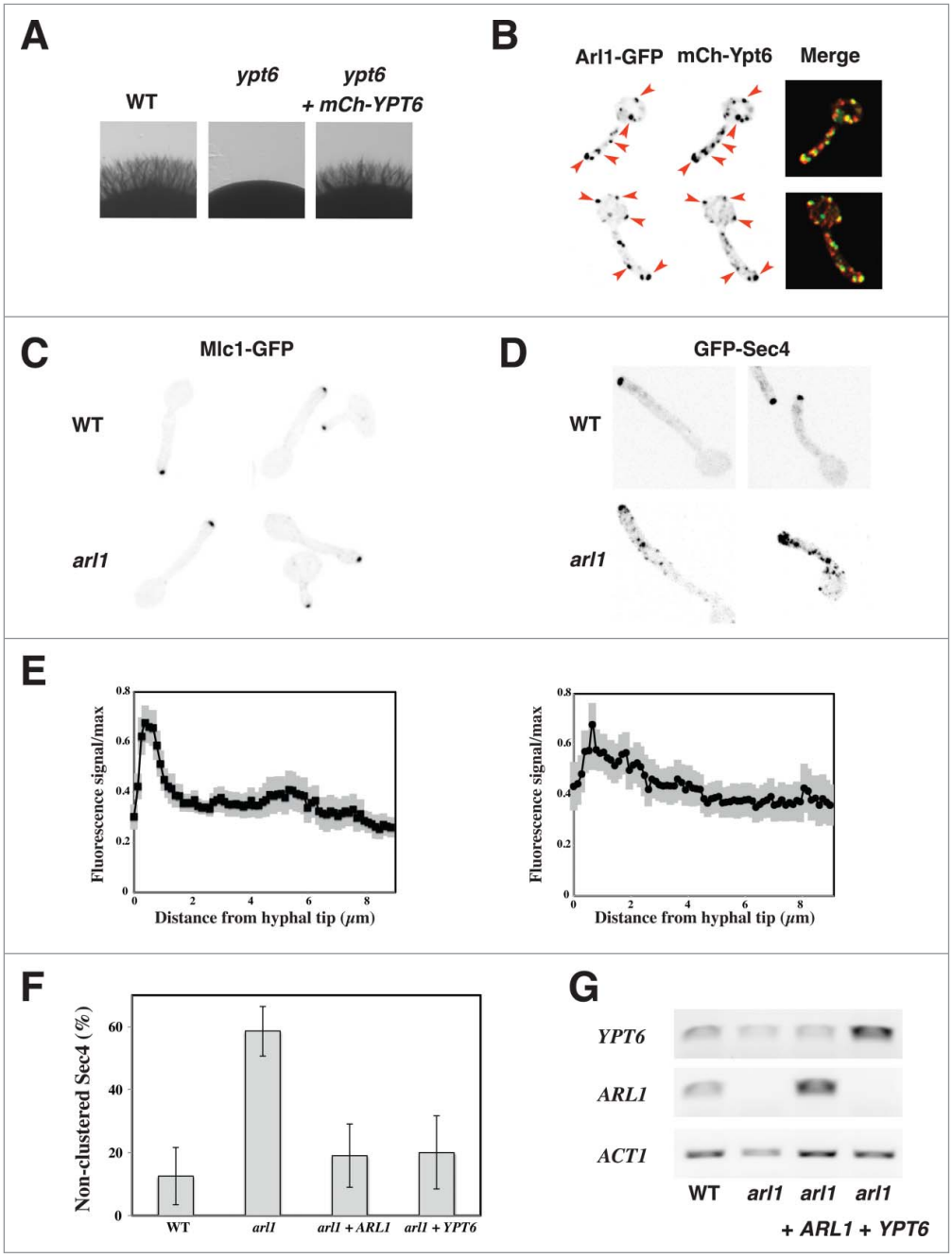

Figure 2. Overexpression of *YPT6* rescues the altered Sec4 distribution in the *arl1* deletion mutant. (A-B) Arl1 and Ypt6 partially co-localize in hyphae. The mCh-Ypt6 fusion is functional (A) Cells from the indicated strains were incubated on Spider media and images were taken after 5 days. Similar results were observed in 2 independent experiments. Maximum projections of 21 deconvolved z-sections of representative cells expressing Arl1-GFP together with mCh-Ypt6 after 90 min FCS-induced hyphal growth; GFP and mCherry signals were acquired simultaneously. (B) (C-E) Sec4, but not Mlc1, distribution is altered in *arl1/arl1* cells. WT and *arl1/arl1* cells, expressing Mlc1-GFP (C) or GFP-Sec4 (D), were incubated with FCS for 45 and 90 min, respectively. Representative images are shown. Cell fluorescence concentration profiles were analyzed with the HyphalPolarity program,[47] to quantify the GFP-Sec4 signal concentration along the major axis of hyphal cells, from sum projection images generated with Image J (E). (F) Overexpression of *YPT6* restores Sec4 clustering in *arl1/arl1* cells. The graph shows averages of 3 independent experiments (n = 10–30 cells each) for each indicated strain, with p values: *arl1 vs* WT: 0.0004, *arl1* + *ARL1 vs arl1*: 0.0028 and *arl1* + *YPT6 vs arl1*: 0.0042; no statistically significant difference was observed between the values for WT, *arl1* + *ARL1* and *arl1* + *YPT6*. (G) *YPT6* and *ARL1* transcripts in strains expressing GFP-Sec4. Transcripts were determined as in Fig. 1F.

over-expressing *YPT6* have an average hyphal length of 16 ± 3 μm (n = 400 cells), which is intermediate compared to 12 ± 2 μm (n = 250 cells) and 21 ± 4 μm (n = 160 cells) for the *arl1/arl1* and the *arl1/arl1* over-expressing *ARL1* cells, respectively. Over-expression of *YPT6* in WT cells did not result in an increase of hyphal length (21 ± 4 μm, n = 300 cells, compared to 23 ± 4 μm, n = 200 cells, for the WT cells).

Furthermore, the *Δ/pTetARF2* mutant, and to a lesser extent *arl1/arl1* mutant, had reduced growth on the cell wall perturbant congo red (CR) a defect complemented by the addition of the respective gene.[18] Fig. 1G shows that the *arl1/arl1* defects on this cell wall perturbant is not rescued by the over-expression of *YPT6*. In *C. albicans*, cell wall integrity mutants often exhibit a growth defect on hygromycin B (HygB). In *S. cerevisiae*, the hypersensitivity of an *arl1* deletion mutant to hygromycin B was not rescued by *YPT6* overexpression.[21] Fig. 1G shows, intriguingly, that the growth defect on hygromycin B was stronger for the *arl1/arl1* than the *Δ/pTetARF2* mutant and, in contrast to growth on CR, the growth defect of the *arl1/arl1* mutant was rescued by over-expression of *YPT6*.

Previously, we showed that a functional Arl1-mCh fusion localizes to the late Golgi by colocalization with Sec7-GFP.[18] Here, we generated a functional mCh-Ypt6 fusion, which complements the invasive growth defect of the *ypt6/ypt6* mutant (Fig. 2A). In *S. cerevisiae* budding cells, Ypt6 was shown to localize both to the cis- and trans-Golgi, with ~ 30% colocalization with Sec7,[27] while in *Aspergillus nidulans*, its homolog RabC also localized to the Spitzenkörper.[28] Comparison of the localization of mCh-Ypt6 to that of Arl1-GFP in *C. albicans* WT cells (Fig. 2B) revealed that the majority of Arl1 containing punctae also had Ypt6 signal (67 ± 8% punctae had both signals, $n = 55$ cells), indicating that Arl1 and Ypt6 are largely co-localized during hyphal growth. To confirm this result, we compared the localization of Ypt6 to that of Sec7 and determined a similar level of colocalization (63% ± 4% of Ypt6 containing punctae had Sec7 signal, $n = 90$ cells).

Our previous results suggested that the polarized growth defect in the *arl1/arl1* mutant results from misregulated secretion.[18] We further investigated secretion in this *arl1/arl1* mutant by examining the distribution of a Spitzenkörper marker, the myosin light chain, Mlc1, and that of secretory vesicles, the small Rab G-protein Sec4.[7] Fig. 2C shows that Mlc1 was clustered similarly in the *arl1/arl1* and the WT hyphal cells and quantification of the fluorescent signal at the tip indicated that it was comparable in the two strains, suggesting that the Spitzenkörper is not substantially altered. In contrast, Fig. 2D shows that the distribution of Sec4 was altered in the *arl1/arl1* mutant, compared to the WT. During hyphal growth, Sec4 is predominantly clustered at the tip of the hyphal cell in *C. albicans*, as well as in *A. nidulans*[7,29] which was reflected by the distribution profiles in the WT cells, shown in Fig. 2E. In comparison, the Sec4 distribution in the *arl1/arl1* cells was less tip-clustered. As illustrated in Fig. 2F, there was a higher percentage of *arl1/arl1* cells exhibiting non-clustered secretory vesicles, compared to the WT cells and the mutant over-expressing *ARL1*, 58 ± 8% compared to 12 ± 9% and 19 ± 10%, respectively. Interestingly, the WT Sec4 distribution in the *arl1/arl1* mutant was restored by over-expression of *YPT6*, with only 20 ± 12% of cells with non-clustered secretory vesicles. Similar to that reported previously,[18] the filament extension rate in the *arl1/arl1* cells expressing GFP-Sec4 was slightly reduced compared to that of WT cells expressing GFP-Sec4 (12 ± 2 μm/h in *arl1* compared to 15 ± 2 μm/h in WT). Overexpression of either *ARL1* or *YPT6* in this *arl1/arl1* strain background restored the filament extension rate to that of the WT (19 ± 3 μm/h and 15 ± 2 μm/h, respectively) suggesting that the alteration in Sec4 distribution is associated with reduced filament extension rate. Interestingly, we observed that the *ypt6* mutant also had a reduced filament extension rate (in preparation), similar to what was observed in *A. nidulans*.[28]

This study revealed that overexpression of the RAB6 homolog Ypt6 can rescue the hyphal growth defect and secretory vesicles clustering defect of an *arl1* mutant. In HeLa cells, RAB6 regulates the movement and docking of secretory carriers to the plasma membrane[30] and in specialized cell types, such as macrophages and developing neurons, this RAB6-dependent secretory pathway fulfills specific functions.[31,32] In *A. nidulans*, deletion of the Ypt6 homolog, RabC, also resulted in impaired secretion.[28] An attractive possibility is that the increased level of Ypt6 facilitates membrane traffic in the *C. albicans arl1* mutant, perhaps *via* promoting targeting of secretory vesicles to the growth site. Alternatively, as the *arl1* deletion mutant has a reduced hyphal extension rate, it is also possible that the alteration of Sec4 distribution results indirectly from reduced growth rather than from Arl1 regulation. Hence, over-expression of Ypt6 could rescue such a defect as a result of increasing the hyphal extension rate. In such a scenario, we can imagine that the Arl1 and Ypt6 genetic interaction during retrograde vesicular transport *via* the GARP (Golgi-associated retrograde protein) complex, observed in *S. cerevisiae*[33–35], is critical specifically for hyphal growth, perhaps for lipid homeostasis.[36] Further characterization of the *arl1* and ypt6 mutants will be necessary to define the specific roles of these small GTPases during hyphal growth. *C. albicans* has only 9 Rab proteins, compared to nearly 70 in mammalian cells and 11 in *S. cerevisiae*. Indeed, homologs of *S. cerevisiae* Ypt10 and Ypt11 and homologs of Ypt4 (Human Rab4 homolog), present in filamentous fungi such as *A. nidulans*, are not present in *C. albicans*. Hence, *C. albicans* has the minimal protein trafficking machinery[37] and identification of the regulators and effectors of critical Rab and Arf GTPases during transition between yeast and hyphal growth should shed light

on the key elements of membrane traffic, sufficient to achieve morphological changes.

Materials and methods

Growth conditions

Yeast extract-peptone dextrose (YEPD) was used and strains were grown at 30°C, unless indicated otherwise. Filamentous growth induction was carried out as described previously either with 50% serum[38] or Spider medium.[39] Congo red and Hygromycin B were from Fluka, Sigma-Aldrich, Saint Quentin Fallavier, France.

Strains and plasmids

Strains used are listed in Table S1. All strains were derived from BWP17.[40] The *ypt6Δ/ypt6Δ* strain was generated by homologous recombination. Each copy was replaced by either *HIS1* or *URA3*, using knockout cassettes generated by amplification of pGem*HIS1* and pGem*URA3*.[40] Primers with a unique RsrII, at the 5′ end, and a unique MluI, at the 3′ end, were used to amplify the *YPT6* and *ARL1* ORFs, and the fragments subsequently cloned into pExpArg-pADH1RAC1,[41] yielding to pExpArg-*pADH1YPT6* and pExpArg-*pADH1ARL1*, respectively.

pExpArg-*pADH1YPT6[Q71L]* and pExpArg-*pADH1YPT6[T25N]* were generated by site-directed mutagenesis of pExpArg-*pADH1YPT6*. The GFP-Sec4 expressing strains and the Mlc1-GFPγ expressing strains were generated as described.[42,43] pExpArg-*pYPT6mChYPT6* was constructed by cloning yemCherry (yeast enhanced monomeric Cherry), amplified by PCR from pFA-yemCherry plasmid using primer pairs with unique Pac1 site, into pExpArg-*pYPT6YPT6*. pExpArg-*pYPT6YPT6* was constructed by amplifying from gDNA *YPT6* ORF with 1 kb upstream and downstream, using primer pairs with unique Xho1 and Not1 sites at the 5′ and 3′ ends; pExpArg-*pARF2ARF2*,[18] with unique Xho1 and Not1 sites, was used to subclone the PCR amplified fragment.

All pExpArg plasmids were linearized with StuI and integrated into the RP10 locus. Two independent clones of each strain were generated and confirmed by PCR. All PCR amplified products and site-directed mutagenesis products were confirmed by sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Microscopy analyses

For colony morphology analyses, plates were incubated for 3–6 days prior to imaging. For cell morphology studies, cells were imaged by differential interference contrast. For GFP-Sec4 imaging, z-stacks were acquired as described.[8] For Arl1 and Ypt6 co-localization experiments, GFP and yemCherry signals were acquired simultaneously and the analyses were carried out as described.[8] Images were deconvolved, using Huygens Professional software (V3.7)

General techniques

RT-PCR analyses were carried out as described.[44,45] Genomic DNA from *C. albicans* strains was isolated as described.[46]

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