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Article Addendum

Bacterial peptidoglycan-derived molecules activate *Candida albicans* hyphal growth

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Serum strongly induces the yeast-to-hypha growth transition in the human fungal pathogen *Candida albicans*, playing an important role in infection. However, identity of the serum inducer(s) and its sensor remain poorly defined. We used NMR to analyze the chromatographic serum fractionations enriched for the hypha-inducing activity and found structures resembling subunits of bacterial peptidoglycan (PGN). We then confirmed that several purified and synthetic muramyl dipeptides (MDPs), subunits of PGN, can indeed strongly promote *C. albicans* hyphal growth. Taking cue from the recognition of MDPs by the mammalian bacterial sensor Nod2 using its leucine-rich-repeat (LRR) domain, we discovered that MDPs activate the adenylyl cyclase Cyr1 by binding to its LRR domain. The cAMP/PKA signaling pathway is well known to control hyphal morphogenesis and other infection-related traits. Given the abundance of PGN at the large intestinal epithelial surface, a natural habitat and invasion site for *C. albcians*, our findings have important implications in the mechanisms of infection by this pathogen.

The AIDS pandemic has seen the rapid emergence of *C. albicans* from a largely benign commensal to one of the most deadly pathogens in humans.[1,2] A key virulence trait of this fungus is its ability to switch between yeast and hyphal growth in the host.[3,4] A long held question is: what host molecules trigger the hyphal growth? It has been known for more than 50 years that serum contains potent hypha-inducing activities.[5] Since Feng and colleagues[6] first showed that the active molecules are smaller than 1 kDa, there have been several candidates including proline, methionine and N-acetylglucosamine (GlcNAc).[7-10] However, the concentrations of the two amino acids required for hyphal induction are much higher than what is available in serum, and there has been no report of existence of free GlcNAc molecules in serum.

We performed chromatographic fractionations of human and bovine serum and analyzed by NMR the fractions enriched for the hypha-inducing activity.[11] We found molecules resembling muramic acid (Mur), alanine (Ala) and isoglutamine (iGln). Since Mur and D-iGln are uniquely present in bacterial PGN in nature,[12] we proposed a structure Mur-L-Ala-D-iGln based on the conserved PGN subunit MurNAc-L-Ala-D-iGln. We then synthesized Mur-L-Ala-D-iGln together with several related compounds (Fig. 1A). Test of hyphal induction showed that all the compounds are active. The most active ones are 1,6-anhydro-MurNAc-L-Ala-D-Glu and MurNAc-L-Ala-D-Glu with I_{50}s of 2.5 and 5.8 μM respectively, while MurNAc-L-Ala-D-iGln is the least active with an I_{50} of 4 mM. The initially proposed structure Mur-L-Ala-D-iGln has an I_{50} of 32 μM. For comparison, the I_{50} of GlcNAc is ~1.8 mM. Thus, the data not only confirm that our proposed molecule is indeed an inducer of *C. albicans* hyphal growth but also revealed molecules that are much more potent. We also determined the structural requirement for the activity. While Mur and Ala exhibited no activity either singly or as a mixture, Mur-L-Ala was found to be active with an I_{50} of ~100 μM, which is the smallest molecule we have found so far with significant activity. In addition, the L configuration of Ala is essential, because Mur-D-Ala and Mur-D-Ala-D-iGln had no activity at all.

The most likely source of PGN in blood is the microflora in the human body.[13,14] Due to the heterogeneity of PGN in different bacterial species and many possible ways for PGN degradation, PGN-derived molecules in serum likely exist in diverse forms. What are the levels of these molecules in serum? Can they account for serum's hypha-inducing activity? Using HPLC and mass spectrometry (MS), we detected in multiple serum samples 0.1 to 0.5 μM of Mur, an indication of the minimal amount of Mur-containing compounds. These levels are apparently several times lower than the I_{50} of the most active compound we found. A plausible explanation is that serum contains other unidentified PGN-derived molecules with higher activity than those we have tested. Consistent with this hypothesis, hydrolysis of MurNAc-L-Ala-D-iGln resulted in lysates with an I_{50} of ~40 nM. However, we cannot conclude that the PGN-derived molecules are predominantly responsible for the hyphal-inducing activity in serum because of the lack of means to specifically remove these molecules. The use of germ-free mice was thought to be a solu-

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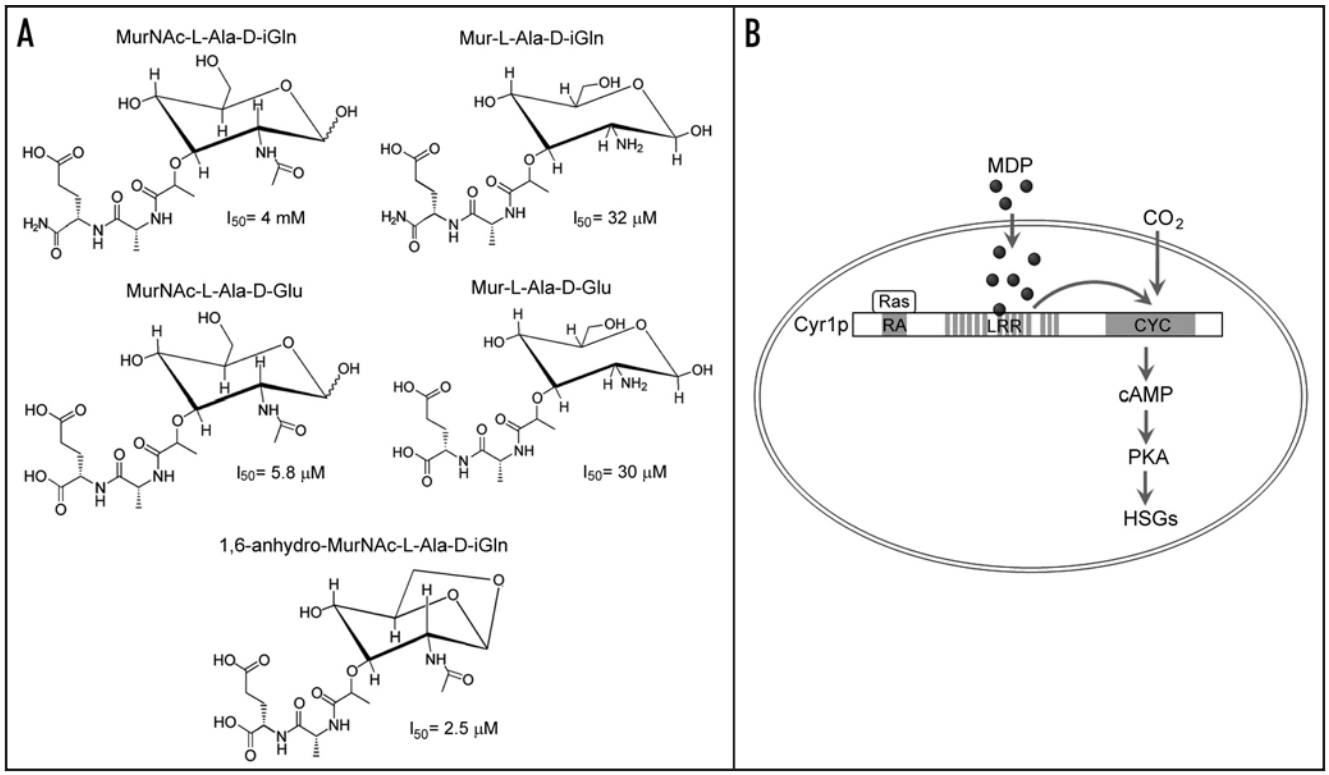

Figure 1. (A) Structure and hypha-inducing activity of several muramyl dipeptides (MDPs). For hyphal induction, 5 x 10^5 yeast cells/ml were incubated at 37°C for 2 hr in Hank's buffered salt solution supplemented with different concentrations of each MDP. I_{50} is the concentration of a MDP that caused hyphal growth in 50% of the yeast cells. (B) Schematic description of the mechanism by which MDPs activate Cyr1 and promote hyphal growth. RA, Ras-association domain; CYC, catalytic domain; HSG, hypha-specifc gene.

tion, but we found that serum samples from such mice are equally active and contain similar levels of Mur as serum from normal mice. It is likely that bacteria killed by sterilization can still enter the body of the germ-free animals through food and water intake.

What is the sensor of PGN in *C. albicans*? Pathogen-associated molecules are recognized by pattern-recognition receptors (PRRs) in diverse organisms.[15,16] PRRs commonly contain a long LRR domain for ligand binding. Using the LRR domain of the mammalian PRR Nod2,[17,18] to search the *C. albicans* genome database revealed 14 LRRs in the adenylyl cyclase Cyr1, a component of the cAMP/protein kinase A (PKA) pathway essential for hyphal development.[19,20] We obtained several lines of compelling evidence strongly supporting the idea that the LRR domain of Cyr1 acts as a sensor of MDPs. First, deleting the entire or part of the LRR domain abolished the cAMP spike that normally occurs following hyphal induction without affecting the cAMP level in yeast cells. Second, these mutants are defective in hyphal growth. Third, MDP conjugated-beads could specifically pull down the Cyr1 LRR domain expressed in *E. coli* and Cyr1 from *C. albicans* cell lysates. Cyr1 is an intracellular protein, indicating that MDPs must first cross the plasma membrane. Using H^3-labelling and MS, we detected intact MDPs in the cell, although the mechanism of transport remains unknown.

In summary, we discovered that PGN-derived molecules in serum are strong inducers of *C. albicans* hyphal growth. We propose that these molecules enter the cell, bind to the LRR domain of the adenylyl cyclase Cyr1 and enhance cAMP synthesis (Fig. 1B). Our data suggest that Cyr1 is a sensor/effector molecule. But do other hyphal-inducing molecules activate Cyr1 by a similar mechanism? CO_2 has been shown to directly act on the catalytic domain of Cyr1,[21] while the GTPase Ras1 has been shown to activate Cyr1 by binding to the Ras-association domain.[22,23] Thus, it remains to be elucidated how Cyr1 receives and responds to different hypha-inducing signals leading to proper hyphal morphogenesis.

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