

Proteome Biology of *Candida albicans* Infection and Action Mechanism of Antifungal Agents

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Introduction

Candida albicans is a common opportunistic pathogenic fungus and its virulence is largely attributable to its unique reversible dimorphic transition from non-pathogenic yeast cells to filamentous hyphal cells. We have previously identified a novel protoberberine-derivative known as HWY-289, which has fungicidal activity against not only *C. albicans*, but also azole-resistant isolates of *Candida* sp. However, the exact molecular mechanism underlying the antifungal effect of HWY-289 is not clear. Here, we describe comparative proteomic analysis of hyphal forms of HWY-289-exposed *C. albicans* and those proteins that were differentially expressed during dimorphic transition from yeast to hyphal cells. In addition, proteomic analyses of macrophage cell lines infected with *C. albicans* using both 2D gel and 2D-LC PF2D system will also be discussed.

Methods

C. albicans filamentous growth was induced with serum-containing medium or spider medium as described elsewhere. A subset of cultures was treated with the indicated concentrations of HWY-289 to inhibit morphological transition. Cells were stained with calcofluor white and visualized using a Zeiss (Germany) fluorescence microscope equipped with a Spot Digital Camera (Diagnostic Instruments, MI). Cultures were treated with 6 mg/L HWY-289 (final concentration) or an equivalent volume of DMSO, collected by centrifugation at $2,500 \times g$ for 5 min at room temperature and resuspended in a analysis buffer.

Results

Among the proteins undergoing transition-associated changes in expression, only those that could be detected in yeast and hyphal forms both in the presence or absence of HWY-289 were analyzed. To analyze protein expression in hyphae, we transferred cells to hyphae-inducing medium and then incubated in 6 mg/L HWY-289 for 9 h. 2-DE of protein extracts revealed that, of the 24 protein spots detected in yeast cells treated with or without HWY-289, only 11 were also detected in hyphal cells treated with or without HWY-289. These 11 proteins could be divided into four groups (Class A-D) based on their expression pattern. Class A proteins were induced during yeast-to-hyphae transition and were suppressed by HWY-289 in both fungal forms. These proteins included CaAcolp and CaSec14p. The ability of HWY-289 to down regulate expression of these hyphal transition-associated proteins suggests that they are critical for virulence of *C. albicans*. These two proteins might account, at least partly, for the antifungal action of HWY-289. The class B proteins, CaAox1p and CaSou1p, were down regulated during hyphal transition regardless of the presence of HWY-289, suggesting that they belong to a group of HWY-289-response suppressed proteins. The Class C proteins, CaEnolp and CaMdh1p, were suppressed during hyphal

transition, as previously reported. Moreover, HWY-289 induced expression of these proteins in both yeast and hyphal cells. The finding that these proteins were induced by HWY-289 in cells that were grown in hyphal-induction medium suggests that they belong to a group of HWY-289-response induced proteins. The class D proteins included CaAde1, CabBmh2p, and CaEpb1p. These proteins were up regulated during hyphal transition regardless of the presence of HWY-289, suggesting that they are HWY-289-resistant hyphal-inducing proteins. Although a small number of proteins were analyzed here because of technical limitations, the expression patterns of these protein classes suggest that multiple types of hyphal transition-related proteins regulate dimorphic transition of *C. albicans* in response to HWY-289. This information will be useful for designing and evaluating potential antifungal drugs that target suppression of virulent filamentous forms of infectious fungi.

Innovative aspects)

- This proteomic analysis has identified potential molecular targets of HWY-289, which include key proteins involved in both cellular energy metabolism and antioxidant defenses.
- HWY-289 may also suppress hyphae formation by down regulating RAS genes which are critical for *C. albicans* hyphal transition.
- Thus, interruption of this pathway may underlie the antifungal activity of HWY-289.

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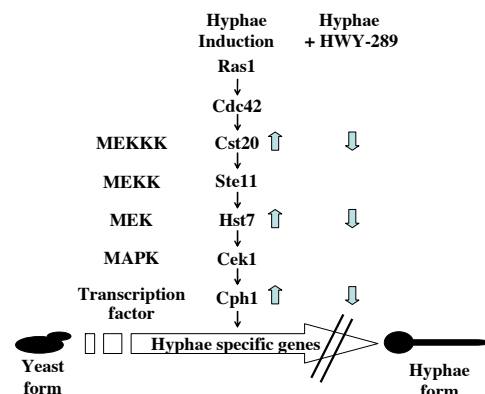


Figure 1. Proposed action of HWY-289 on the RAS signaling pathway and *C. albicans* hyphal transition. Arrows indicate the up regulation (↑) or down regulation (↓) of gene expression observed here. This diagram was adapted from a figure published by Liu, H. *et al.* (1994).

Proteomics Applied to the Study of Hepatitis C Virus Pathogenesis

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Introduction

Hepatitis C virus (HCV) often causes persistent infection in humans, a serious condition that is associated with chronic liver disease, cirrhosis and hepatocellular carcinoma, and represents a major issue of public health worldwide. For years, HCV research has been hampered by the lack of a robust cell culture system that recapitulates the complete viral life cycle. These limitations have been recently overcome by the use of the JFH genotype 2a strain of HCV, which permits propagation of infectious HCV particles in cell culture at high yields.

Methods

We used this cell culture system to comprehensively characterize the protein changes associated with HCV infection upon a 4 months period and identify host proteins involved in HCV life cycle. In addition, the particles produced in this system have been shown to be infectious *in vivo*. We carried out a proteomic analysis on highly purified HCV J6/JFH virions to the characterization of the composition of the HCV virion. identify host proteins associated with the released viral particles.

Results

Proteomic profiling based on SDS-PAGE and LC-MS/MS was performed on HCV infected cells at 6 time points over 5 months, corresponding to early infection, peak of infectivity and viral replication, massive cell death and virus clearance and finally chronic phase of infection. The analysis identified sets of host proteins specifically involved in each of these phases of infection. In addition, viral particles were purified on sucrose gradients and analyzed by a similar proteomic approach. The analysis of the released viral particles identified proteins associated with the virion. These results were further validated by immunogold electron microscopy performed on viral particles from an HCV genotype 2a-infected patient. Pre-incubation of HCV particles with targeted antibodies decreased viral infectivity and reduction of targeted protein expression by an RNAi approach decreased viral release without affecting HCV replication levels. These results suggest that these identified proteins provide HCV with means for cell entry and release.

Innovative aspects

- Application of proteomics technologies to study virus particles
- Proteomics-based discovery for novel antiviral strategies as well as vaccinotherapy

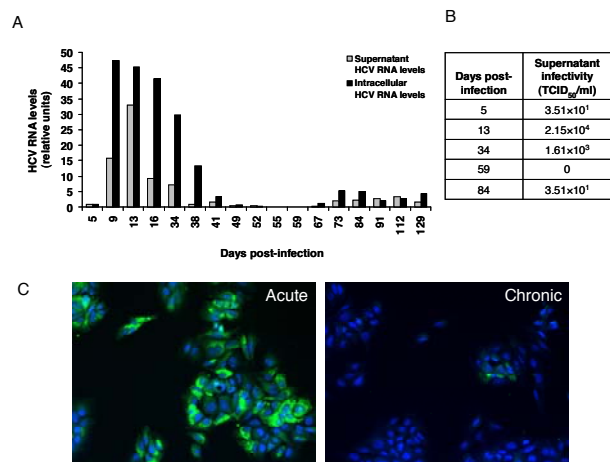


Figure 1: Levels of HCV viral replication and infectivity over 4 months post-infection. Infected cells representative of 1- early infection, 2- peak of viral replication and infectivity, 3- induction of innate response, 4- virus clearance and 5- chronic phase, were collected at 5, 13, 34, 59 and 84 days post-infection, respectively. The proteomic profiling of the infected cells at these different time points will be presented.

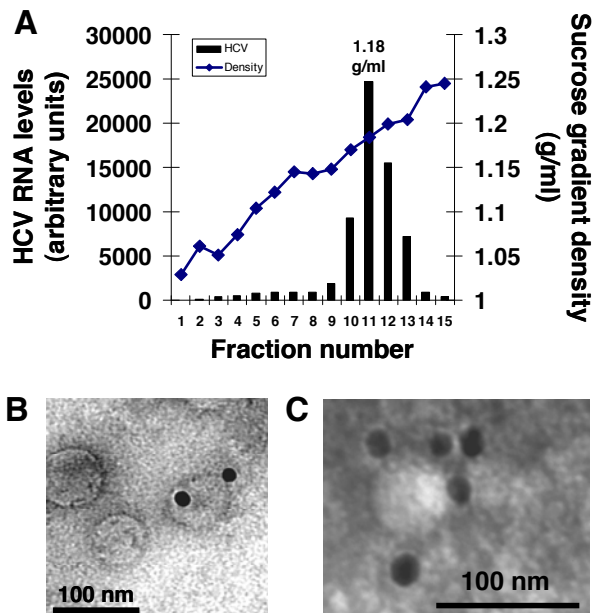


Figure 2: Identification of host proteins associated with HCV particles. Purification of viral particles (A) for proteomic analysis and validation by electron microscopy of the association between one of these identified host protein with viral particles from the culture system (B) and with particles isolated from an infected patient (C).

Proteolytic processing of the Campylobacter adherence Factor (CadF) in *Campylobacter jejuni*

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Introduction

Campylobacter jejuni is the leading cause of bacterial gastroenteritis in the developed world. Colonization of *C. jejuni* is mediated by binding to the extracellular matrix (ECM). Campylobacter adherence Factor (CadF) is an outer membrane protein responsible for the binding of fibronectin to the ECM [1]. We compared the outer membrane protein profiles of two *C. jejuni* isolates, and identified the presence of multiple CadF variants. MS analysis of these suggests that CadF undergoes proteolytic processing within clinical strains that results in reduced immunogenicity.

Methods

The outer membrane protein profiles of two *C. jejuni* isolates; one isolated from a patient with typical gastrointestinal symptoms (JHH1) and the second, a passaged ex-virulent strain (ATCC700297), were compared using 2-DE, Western blotting and mass spectrometry [2]. Several spots corresponding to CadF were detected in each isolate. We utilized peptide mass mapping on a Voyager DE-STR MALDI-TOF MS and MS/MS using an Applied Biosystems QSTAR xl instrument to compare peptide profiles and sequences from each of these variants. Whole protein MS was carried out using MALDI-MS to confirm the intact protein masses. Fibronectin binding capacity was confirmed using fibronectin ligand blotting.

Results

Multiple spots corresponding to CadF were detected in each isolate, however two series of lower mass variants were only identified in the clinical isolate JHH1. Peptide mass mapping of the multiple variants revealed that these lower mass forms corresponded to the N-terminal 20-22kDa of native CadF. This loss of the C-terminus was confirmed by intact protein MS and the site of cleavage elucidated. Analysis of the CadF amino acid sequence revealed that the C-terminus corresponded to an OmpA domain that has been previously shown to be immunogenic within *Salmonella enterica* [3]. Probing of the CadF fragments against convalescent patient serum revealed that these lower fragments were no longer immunogenic but still retained the ability to bind fibronectin as determined by fibronectin ligand blots. These results suggest that the immunogenic epitope of CadF is located within the C-terminus and that removal limits recognition of CadF by the humoral immune response.

Innovative aspects

- Identification of multiple CadF variants that are proteolytically processed and are likely to be involved in binding of host epithelium while limiting the immune response.
- Mass spectrometry was able to identify the cleavage sites in both lower mass forms as well as prove several other variants resulted from re-folding during analysis.
- Proteolytic processing is an important, yet neglected, protein post-translational modification.

References

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Proteomics of influenza virus and vaccine.

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Introduction

Influenza virus is one of the most prevalent and highly contagious pathogens, which continues to have a significant impact on today's society with over half a million deaths and up to 5 million annual hospitalizations worldwide every year (1). Despite the fact that this virus is thoroughly studied and there is a good understanding of its genome, replication, structure and function of its proteins, the disease is at best managed rather than controlled due to constant antigenic changes in two major viral coat proteins, haemagglutinin (HA) and neuraminidase (NA) of the circulating influenza A (subtypes H1N1 and H3N2) and influenza B viruses. These changes result from the accumulation of point mutations in the genes that encode the two proteins (termed "antigenic drift") and lead to the constant emergence of new virus strains against which there is little or no pre-existing immunity in the population (2). The single radial immunodiffusion (SRID) assay is currently used to identify the virus strain included in an influenza vaccine preparation, as well as to determine vaccine potency (3). In order to perform this essential test, HA must first be purified and then used to immunize sheep for the production of HA-specific antiserum. This poses a potential bottleneck for vaccine manufacture that is of particular concern for the generation of SRID reference reagents and antisera for pandemic H5N1 vaccines. In addition, the test can be influenced by the physical state of the vaccine preparation (e.g. Tween-ether split vs. monovalent whole virus); it does not provide any information concerning presence of other viral proteins and possible non-viral protein contaminations; and it cannot distinguish between drift variants of different strains. Alternative proteomics-based assays that are independent of such reagents would prevent this potential bottleneck.

Results

This work reports on an ongoing effort to develop a mass-spectrometry (MS)-based technique that provides both qualitative and quantitative analysis of different types of influenza virus and monovalent and trivalent influenza vaccine preparations. First, a peptide list was generated from in silico tryptic digest of HAs from influenza A subtypes H1N1 (A/New Caledonia/20/99), H3N2 (A/Wisconsin/67/2005), and influenza B (B/Ohio/01/2005), the strains that compose the 2006/07 Northern hemisphere vaccine, using protein sequences from the influenza virus database (<http://influenza.genomics.org.cn/>). Next, the list was matched with that of the mass spectra

data obtained from MALDI MS analysis of the tryptic digest of purified recombinant HAs from each of the aforementioned virus types. In silico and tryptic peptides of HA from H3 drift variants, A/Wisconsin/67/2005, A/New York/55/2004, and A/Wyoming/3/03 were also compared. On the basis of this information and published data regarding antigenic sites of HA, peptides from each virus subtype were selected for qualitative characterization of vaccines. In addition, a peptide that is unique to each H3 variant was identified. The hypothesis that the selected peptides will identify a virus subtype as well as the specific strain contained in a trivalent influenza subunit vaccine was tested using a 2006/2007 formulation manufactured by Sanofi Pasteur Inc. (Swiftwater, PA). The vaccine was digested with trypsin and the resulting peptide mixture was analyzed using MALDI TOF/TOF instrument. The results showed the presence of subtype and strain specific peptides, confirming the presence of A/New Caledonia/20/99 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004 in the vaccine preparation. Our current experiments aim to use the amount of peptide present to determine HA quantity, with the goal of establishing a method to measure the absolute amount of HA in a vaccine or reference antigen preparation. This is an essential first step in evaluation of vaccine potency.

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"The findings and conclusions in this presentation have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy."

Phosphoproteome of Primary Human Host Cells Infected with *Yersinia pestis*

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Introduction

Yersinia pestis is a Gram-negative bacterium that causes plague. Upon cell contact, *Y. pestis* injects several virulence proteins that disrupt function and survival of the host cell (1). Two of these factors that are essential for virulence are modulators of host protein phosphorylation: the tyrosine phosphatase YopH and the protein kinase YpkA. We describe the results of a comparative proteomic analysis of phosphorylation changes in primary cultures of human monocytes to characterize the multitude of changes in the host cell phosphoproteome resulting from infection with *Y. pestis*.

Methods

CD14+ human monocytes were immunoaffinity purified from donor blood samples (98% purity) and were infected with an attenuated strain of *Y. pestis*. Protein extracts from both uninfected control and infected samples were prepared at several time points following initiation of infection, and were comparatively profiled using quantitative data-dependent neutral loss nano-RPLC-MS²-MS³ analysis. For all samples, both Gallium resin enriched phosphopeptides and the phosphopeptides recovered from the non-bound fraction were subjected to quantitative profiling using ¹⁶O/¹⁸O labeling followed by SCX-based fractionation (2). These results were analyzed by database search using the SEQUEST program and the phosphorylated peptides thus identified were manually verified.

Results

We identified host cell proteins that undergo significant phosphorylation changes as a consequence of infection, providing new insights into the etiology of plague at a molecular level. A total of approximately 120 phosphoproteins were identified from each time point of infection, and from each data set the phosphorylation profile of about 90 proteins were quantified with a high degree of confidence. These results allowed the identification of several novel targets that exhibited significant phosphorylation changes as a consequence of time from infection and the specific protein segments that were phosphorylated or dephosphorylated in these proteins.

Innovative Aspects

- Application of highly purified primary cultures of human monocytes and use of a well-characterized cell culture infection model for the proteomic analysis to reduce false positives and irrelevant phosphorylation events due to host cell heterogeneity or immortalizing transformation.
- Quantitative profiling of phosphorylation changes in primary (non-transformed) human host cells following *Y. pestis* infection
- Proteomic analysis of time-dependent phosphorylation changes in host cell proteins induced by *Y. pestis* infection

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