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The Influence of Histatin 5 on *Candida albicans* Mitochondrial Protein Expression Assessed by Quantitative Mass Spectrometry

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Abstract

Individual aspects of the mode of action of histatin 5, a human salivary antifungal protein, have been partially elucidated, but the mechanism likely involves a complex set of events which have not been characterized. Previous evidence points toward histatin-induced alterations in mitochondrial function. The purpose of the present study was to verify and quantify changes in the mitochondrial proteome of *C. albicans* treated with histatin 5. Cell killing was determined by plating and differential protein expression levels in the mitochondrial samples were determined by quantitative proteomics approaches employing mTRAQ and ICAT labeling and Western blotting. Relative quantitation ratios were established for 144 different proteins. Up-regulated mitochondrial proteins were predominantly involved in genome maintenance and gene expression, whereas proteins that constitute the respiratory enzyme complexes were mostly down-regulated. The differential expression of ATP synthase gamma chain and elongation factor 1-alpha were confirmed by Western blotting by comparison to levels of cytochrome c which were unchanged upon histatin treatment. The mTRAQ and ICAT proteomics results suggest that key steps in the histatin 5 antifungal mechanism involve a bioenergetic collapse of *C. albicans*, caused essentially by a decrease in mitochondrial ATP synthesis.

Keywords

Histatin; *Candida albicans*; mitochondrial proteome; quantitative proteomics; mTRAQ; ICAT; antifungal mechanism

Introduction

Candida albicans is the most prevalent species among the fungi pathogenic for man and represents an important opportunistic microorganism in superficial infections of the

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Supporting Information Available: Supplemental Table. This information is available free of charge via the Internet at http://pubs.acs.org.

oropharyngeal mucosa.1 *C. albicans* occurs naturally in about 50-60% of the human population, and becomes pathogenic when the host immune defense system is compromised.2,3 Despite the advent of azole-based antimycotic drugs, the world-wide prevalence of oropharyngeal candidiasis continues to be high. This is mostly due to an increasing population of immuno-compromised individuals, comprising HIV infected patients, transplant recipients, and cancer patients.4-8 Treatment of candidiasis has relied strongly on the use of only a small number of antifungal agents. In addition, the frequent use of the few available antifungal formulations has led to the considerable emergence of antimycotic fungal resistant strains stimulating the search for novel antimycotic drugs with alternative antifungal targets.2

Interestingly, several naturally occurring peptides and proteins have been characterized with significant antifungal activities. Among these are histatins, a family of small histidine-rich cationic proteins of salivary acinar cell origin. The major histatins are histatin 1, 3 and 5 exhibiting molecular weights of 4929 Da, 4063 Da, 3037 Da, respectively.9 The most potent histatin is histatin 5 exhibiting fungicidal activity against *C. albicans* and other fungal species, including *Cryptococcus neoformans*, and *Aspergillus fumigatus* at physiological concentrations of 15 to 30 μM.9-11 While histatins clearly lack any toxicity for human cells, their significant antifungal activities make them attractive candidates as templates for the development of new antifungal formulations.12

The elucidation of the unique antifungal mechanistic pathways of histatin 5 could revolutionize the way in which antifungal drug therapies are designed. To date, fragmented information is available related to the mechanism of action of antimicrobial peptides.13 For a number of these peptides, amphipathicity, induction of helical conformations in hydrophobic environments and transmembrane pore-formation have been shown to be key steps in their antimicrobial mechanisms.14-18 Histatin 5, however, is only weakly amphipathic and no evidence for transmembrane pore formation could be ascertained.19 The evidence gathered so far suggests that histatin 5-provoked killing of *C. albicans* comprises a multistep process with many missing links and uncertainties. For instance, there are at least two possible pathways for histatin 5 translocation from the extracellular space to the cytosol. In one of the models, translocation is driven by the transmembrane potential.20 In the alternative model, histatin 5 binds to specific receptor(s) on the cell surface.21-23. There is more agreement on the mechanism leading to the terminal phases of cell demise, consisting of the rapid efflux of cellular ATP and other nucleotides such as NAD+, AMP, ADP, and small ions.24,25 A fascinating observation made in conjunction with the histatin 5 antifungal mechanism relates to altered mitochondrial function. 20,24,26 Previous studies have shown that once histatin 5 is internalized in the cell, it targets the energized mitochondrion leading to a loss of the mitochondrial transmembrane potential and the generation of ROS by inhibition of the respiratory chain, possibly at the co-enzyme Q level.20,26 Even though some aspects of the mode of action of histatin 5 have been elucidated, the mechanism likely involves a complex set of events which have not yet been characterized and integrated into a comprehensive model.

In view of ample evidence for the importance of cell metabolic activity in the histatin killing mechanism, we aimed to characterize the changes induced by histatin 5 at the mitochondrial level in more detail. This study is geared towards a comprehensive examination of histatin 5-induced protein expression changes at the mitochondrial level. We performed a large scale comparison of such changes using quantitative mass spectrometry employing Amine-Modifying Labeling Reagents for Relative and Absolute Protein Quantitation (mTRAQ) and Isotope-Coded Affinity Tags (ICAT). The ultimate goal is to achieve an in-depth understanding of bioactivity at the molecular level which will provide opportunities to develop mechanism-based novel antimycotics.

Materials and Methods

Materials

Sabouraud dextrose agar (SDA) and Sabouraud dextrose broth (SDB) were purchased from DIFCO (Detroit, MI); $MgCl_2$, sorbitol, dithiothreitol (DTT), mannitol, SDS (sodium dodecyl sulfate), 6N HCl, amino acid standards, iodoacetamide, TPCK-treated trypsin, and sucrose from Sigma (St. Louis, MO); potassium phosphate (K_2HPO_4, KH_2PO_4), imidazole, Tris, phenol, ammonium bicarbonate (NH_4HCO_3), formic acid (HCO_2H), HCl, EDTA, glycine, methanol, Tween®20, and NaCl from Fisher Scientific (Waltham, MA); acetonitrile (CH_3CN) from EMD chemicals (Gibbstown, NJ); Zymolase 100T from Seikagaku (Tokyo, Japan); EGTA (ethylene glycol bis (â -amino ethyl ether)-N, N, N, N-tetraacetic acid) from Eastman (Rochester, NY); L-norleucine from MP Biomedicals (Solon, OH); phenol isothiocyanate (PITC), triethylamine (TEA) and enhanced luminol-based chemiluminescent (ECL) from Thermo Scientific (Rockford, IL); trifluoracetic acid (TFA), amine-reactive tag for relative quantitation (mTRAQ), isotope-coded affinity tags (ICAT), and tris-(2-carboxyethyl)-phosphine (TCPE) from Applied Biosystems Inc. (Foster City, CA); triethylammonium bicarbonate (TEAB) from Fluka (Buchs, Switzerland); polyacrylamide slab gels (12%) from Invitrogen (Carlsbad, CA); polyvinylidene difluoride (PVDF) membrane from Millipore (Billerica, MA); bovine serum albumin (BSA) from Boehringer Mannheim GmbH (Mannheim, Germany); rabbit anti-elongation factor 1 alpha 1 (EEF1A1) polyclonal antibody and rabbit anti-gamma subunit of ATP synthase (AtpC) polyclonal antibody from GmbH (Atlanta, GA); mouse anti-cytochrome c monoclonal antibody 7H8.2C12 (Cytc) from BD Biosciences (San Jose, CA) and sheep anti-mouse IgG-HRP conjugate and sheep anti-rabbit IgG-HRP conjugate from GE Healthcare (Buckinghamshire, UK).

C. albicans culturing and histatin treatment

C. albicans strain SC5314 was grown from a stock culture maintained at -80°C onto SDA for 48 hrs at 30°C. For preparative culturing of *C. albicans*, several colonies were transferred to 1.5 L 10% SDB and cultured to mid-log phase for 16 hrs while shaking at 200 rpm at 30°C. Cells ($OD_{620} \approx 0.8$) were harvested by centrifugation at 15,000×*g* (Sorvall centrifuge RC-5B, Thermo Scientific) at 4°C and suspended in 1 mM potassium phosphate buffer (pH 7.0) to an OD_{620} of 0.8. The suspension was divided into 300 ml aliquots and histatin 5 was added to experimental aliquots at a final concentration of 6 ìM. The concentration chosen represents the IC_{50} value for *C. albicans* cell killing20. Experimental and control suspensions were incubated for 0 min, 30 min or 60 min at 37°C. Cells were centrifuged at 12,000×*g* at 4°C and washed in 1.2 M sorbitol containing 1 mM DTT, 10 mM $MgCl_2$ buffered to pH 7.4 with potassium phosphate. The preparation of spheroplasts was carried out in the same buffer supplemented with Zymolase 100T (1.3 mg/ml) as described previously.20 The efficiency of spheroplast formation was evaluated by measuring cell lysis in deionized water followed by OD measurement at 620 nm.

Isolation of *C. albicans* mitochondria

Mitochondria were isolated from *C. albicans* spheroplasts as described previously.20 Essentially *C. albicans* spheroplasts were collected by centrifugation for 5 min at 1,000×*g* at 4°C, suspended in 0.4 M sorbitol and 10 mM imidazole, pH 6.4, and homogenized manually using a Potter-Elvehjem homogenizer for 5 min on ice. The homogenate was mixed with an equal volume of a solution containg 1 M sorbitol, 25 mM potassium phosphate, 4 mM EGTA and 10 mM imidazole, pH 6.4, and the cell debris was pelleted by centrifugation for 5 min at 1,000×*g* at 4°C. The supernatant was removed and centrifuged for 10 min at 12,000×*g* at 4°C. The reddish pellet containing the mitochondria was suspended in 0.5 ml of

0.6 M mannitol, 2 mM EGTA, and 10 mM imidazole, pH 6.4 and centrifuged again at 10,000×*g* to harvest the mitochondria.

Assessment of the candidacidal activity of histatin 5

After *C. albicans* cells had been incubated with histatin 5 for 0, 30, or 60 min, the percentage cell viability was determined in a killing assay, as described previously.12 Aliquots of 25 μl of the histatin-treated and control cells were diluted in 9 ml PBS, and 25 μl of this diluted cell suspension was plated in triplicate onto SDA. After 48 hrs of incubation at 30°C, candidacidal activity was assessed by counting the number of colony-forming units (CFUs) in the histatin treated and control samples.

Protein concentration determination

The total protein concentrations in the mitochondrial samples were determined by quantitative amino acid analysis as described previously.27 Mitochondrial pellets were dissolved in mitochondrial lysis buffer containing 0.1% SDS and 50 mM Tris, pH 8.5, boiled for 15 min, and stored at -20 °C until used. Briefly, 20 μl of each purified mitochondrial sample was hydrolyzed in the vapor phase by addition of 20 μl of 6 N HCl, containing 1% phenol and incubation for 22 hrs at 108°C. L-norleucine at a final concentration of 10 nmol was added to all samples and used as the internal standard. Amino acids in the hydrolyzed mitochondrial samples and amino acid standards at a final concentration of 12.5 nmol or 6.25 nmol (cysteine: 6.25 nmol and 3.13 nmol) were modified by addition of 20 μl of 10% PITC in 10% TEA, 70% ethanol. After derivatization, an amount equivalent to 10 μl of each sample was applied to a C18 column, at 45°C. The amino acids were separated by RP-HPLC using the Waters PicoTag protocol (Waters, Corp., Milford, MA). Based on the concentration of amino acid standards, the protein concentrations in the mitochondrial fractions were calculated.

mTRAQ labeling and purification of peptides

We used the relatively recently introduced mTRAQ reagent which is a comprehensive isotope labeling reagent reacting with all free amino groups.28 Equal amounts of mitochondrial proteins (100 μg) from experimental and control samples dissolved in mitochondrial lysis buffer were dried using a speedVac (Savant Inc. Irvine, CA) and dissolved in 20 μl of 50 mM TEAB. Reduction of proteins was achieved upon addition of 2 μl of 50 mM TCPE and boiling for 10 min. After alkylation with 7 mM iodoacetamide for 2 hrs, the samples were diluted 10 fold with 50 mM NH_4HCO_3, and mitochondrial proteins were digested with 3% (w/w) TPCK-treated trypsin for 16 hrs at 37°C, and an additional 2% (w/w) for 6 hrs. The samples were dried and dissolved in 100 ìl buffer A containing 2.5% CH_3CN and 0.1% HCO_2H. To avoid interference of amine-containing buffers with the mTRAQ label the peptide digests were purified on C18 Micro Spin column (The Nest Group Southborough, MA). Experimental and control samples were labeled with mTRAQ reagents according to the manufacturer's instructions (Applied Biosystems). The isotopically heavy mTRAQ reagent (148 Da) was used for histain treated samples and the isotopically light label (140 Da) for control samples. After labeling was completed, samples were combined, and excess mTRAQ-reagent was removed using a strong cation exchange cartridge (Phenomenex Torrance, CA). Peptides were subsequently purified using C18 Micro Spin columns (The Nest Group), and dried under vacuum. Three biological experiments were carried out for each incubation time point (0, 30 and 60 min), and each was subjected in triplicate to chemical labeling.

ICAT labeling and purification of peptides

The second approach involved labeling of proteins with isotope coded affinity tags (ICAT) which covalently modify cysteine-containing peptides.29 Equal amounts of mitochondrial protein (100 μg) from experimental and control samples were dissolved and reduced as described above. ICAT labeling of the cysteine residues was carried out according the manufacturer's instructions (Cleavable ICAT® Reagent Kit for Protein Labeling; Applied Biosystems). The isotopically heavy label was used for histatin 5-treated mitochondria and the light reagent was used for control mitochondria. After labeling, the control and the histatin 5 treated samples were combined and proteins were digested with TPCK-treated trypsin as described above. Excess ICAT-reagent was removed using a cation-exchange cartridge (Phenomenex) and ICAT-labeled peptides were purified from the non-labeled peptides using an avidin affinity cartridge (Phenomenex). The peptides were eluted using 500 μl of a buffer containing 0.4% TFA and 30% CH_3CN, and dried. For ICAT labeling, one biological experiment was carried for each incubation time point (0, 30 and 60 min), and each was subjected in triplicate to chemical labeling.

Nano-Flow Liquid-Chromatography and Electrospray-Ionization Tandem Mass Spectrometric (LC–ESI–MS/MS) Analysis

The mTRAQ- and ICAT-labeled samples were suspended in buffer A containing 2.5% CH_3CN, 0.25 % HCO_2H solution and analyzed by nano-flow liquid-chromatography electrospray-ionization tandem mass spectrometry (LC-ESI-MS/MS) (Micro AS, ThermoFinnigan, CA) using a linear ion trap mass spectrometer (Thermo Electron San Jose, CA) equipped with an on-line autosampler (Micro AS). Samples (3 ìl) were injected and loaded on an in-line fused silica microcapillary column (75 ìm in diameter and 100 mm in length) which was packed in-house using C_{18} resin (Micron Bioresource Auburn, CA). Chromatographic separation of peptides was achieved at a flow rate of approximately 200 nl/min over a 55 min time interval using a Surveyor MS Pump Plus (Micro AS). The gradient steps were from 0 to 8% buffer B (containing 97.5% CH_3CN and 0.1% HCO_2H) in 5 min, to 15% buffer B in 10 min, to 25% buffer B in 10 min, to 50% buffer B in 20 min, and to 95% buffer B in 10 min. The eluted peptides were directly nano-electrosprayed and MS/MS data were generated using data-dependent acquisition with a MS survey scan range between 390 and 2000 m/z. Each survey scan (MS) was followed by automated sequential selection of the top five peptides for collision-induced dissociation (CID), at 35% normalized collision energy, with dynamic exclusion of the previously fragmented ions.

Database searches and protein identification

All MS/MS spectra from LC-ESI-MS/MS were searched against the *C. albicans* protein database available at Uniprot (Universal Protein Resource, Version 9.0, http://www.uniprot.org) which combines data from Swiss-Prot (Version 51), TreMBL (Version 34) and PIR. The database searched contained 72746 entries. Searches were carried out using Bioworks 3.3.1 software and the SEQUEST search engine (Thermo-Scientific, San Jose, CA).30›31 The SEQUEST score filter criteria applied to the MS/MS spectra were: precursor-ion tolerance of 1.5 amu, fragment ion tolerance of 1.0 amu, XCorr score ≧ 1.6, 1.8 and 3.0 (for fully tryptic peptides) and 1.8, 2.2 and 3.75 (for partially tryptic peptides) for charge status of Z = 1, 2 and 3, respectively. A ΔCn ≧ of 0.1 and a peptide probability of ≦ 0.5 were applied. Search parameters (modifications) used for mTRAQ were the following: 140 Da static modification on lysine residues and N-termini for peptides labeled with mTRAQ (light) and + 8 Da for dynamic modification on lysine residues and N-termini for peptides labeled with mTRAQ (heavy). Search parameters (modifications) used for ICAT labeled samples were 227 Da static modification on cysteine residues labeled with ICAT (light), and a dynamic modification of + 9 Da for cysteine residues labeled with ICAT (heavy). The relative quantitations of mTRAQ (light and heavy), and ICAT (light and

heavy) were carried out using the pepQuan option in the Bioworks Rev.3.3.1 software and comparing peak areas of isotope-encoded peptide pairs observed in the corresponding MS spectra. For the mTRAQ labeled samples, only proteins for which two or more unique peptides were identified are reported. For the ICAT labeled samples, proteins with single peptide identifications were included since proteins may contain only a single cysteine residue. The obtained protein ratios in a particular sample were normalized to the most frequently observed ratio which was established from a plot of the ratios in a histogram which was created using Excel software. Correction factors (CF) were calculated by dividing 1 by the most frequently observed ratio which was in all cases close to 1 (as expected). CF values were calculated for the total of 9 assays conducted with mTRAQ (3 technical triplicates of 3 biological experiments) and 3 assays conducted with ICAT (3 technical triplicates of one biological experiment). For technical triplicates, the average CF value was used for correction of the data. The biological function of the identified proteins were obtained from UniProt Knowledgebase (UniProtKB) (http://www.uniprot.org/)[32,33] and/or Mitochondrial Proteome 2 (MitoP2) - databases (http://www.mitop.de:8080/mitop2).[34-37] The functional classifications made were based on various studies on the yeast mitochondrial proteome.[34,37]. Grouping of the proteins into 11 functional categories was performed following classifications as published.[33,38,39]

Western Blot Analysis

Western blotting was performed as described previously.[40] Mitochondria were isolated as described above, and were solubilized in SDS sample buffer containing 26.5 mM Tris HCl, 35.3 mM Tris, 0.5% SDS, 2.5% Sucrose, 0.13 mM EDTA, 0.055 mM SERVA® Blue, 0.044 mM Phenol Red and 2.5 mM DTT, pH 8.5. Samples were then boiled for 5 min and stored at -80 °C. Aliquots of these samples were subjected to 12% (v/v) SDS polyacrylamide slab gel electrophoresis (3 μg/lane). The separated proteins were transferred electrophoretically to PVDF membranes for 1 h 30 min at 4°C in blotting buffer containing 103 mM Tris, 139 mM glycine, and 20% (v/v) methanol using blotting equipment (Bio-Rad). Membranes were blocked for 1 h at room temperature with 5% skim milk in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl). The blocking buffer was removed, membranes were washed three times in TBS, and membranes were incubated for 16h at 4°C with the primary antibodies diluted in TBS containing 2.5% (w/v) BSA. The primary antibodies used were a 1:400 dilution of rabbit anti-human elongation factor alpha 1 (EEF1A1) and a 1:10,000 dilution of rabbit anti-*Arabidopsis thaliana* and *Chlamydomonas reinhardtii* chloroplast-localized gamma subunit of ATP synthase (AtpC). The control antibody, used to confirm that equal amounts of protein were present in each lane of the gel, was a 1:250 dilution of mouse-anti pigeon cytochrome c (Cytc). The membranes were subsequently washed three times (10 min/wash) with TBST (TBS containing 0.1% (v/v) Tween-20), and then incubated for 1 h at room temperature with the appropriate secondary antibodies diluted in TBS. Secondary antibodies used were a 1:10,000 dilution of sheep anti-mouse IgG-HRP conjugate and a 1:20,000 dilution of donkey anti-rabbit IgG-HRP conjugate. After incubation with the secondary antibodies, the membranes were washed five times in TBST. The activity of HRP was visualized by incubating the membranes for 5 min at room temperature with ECL solution according to the manufacturer's instructions (Pierce) and luminescence was monitored using X-ray films (Kodak) and a Medical Film Processor (Konica Minolta Medical and Graphic, Inc., Tokyo, Japan). At the end of these experiments, the immuno-detection reagents were removed from the blot followed by three washes in TBST. Membranes were subsequently stripped from bound antibodies by incubating for 45 min at 50°C with Restore Western Blot Stripping buffer (Pierce). The membranes were then re-probed with the second specific primary and secondary antibodies as described above. Densitometric analysis of the radiographic images was performed using a VersaDoc 3000 Imaging System (Bio-Rad). The pixel intensity of the AtpC and the EEF1A1 bands in mitochondria harvested at t=0,

t=30min and t=60 min were normalized to the intensity of the Cytc band in the same samples.

Statistical Analysis

A Student's *t*-test was used to test for significant differences in *C. albicans* cell viability at each time point between control and histatin 5-treated cells. Significant differences in protein expression levels at each time point between control and histatin 5-treated mitochondria were defined by calculating the 95% confidence interval (CI) for both mass spectrometric analysis and Western blot analysis. The CI was determined for proteins for which at least two peptide ratio values were obtained with either of the two labeling methods using the confidence function in Excel software with the following settings; alpha (significance level): 0.05; STDEV: standard deviation of the experimental measurements; size: sample size of the experimental measurements. The value calculated for the 95% CI was subtracted or added to 1.0 and if the average of the experimental ratio did not overlap with the calculated 95 % CI applied to 1.0, the experimental value was considered significantly different from 1.0.

Results

Evaluation of candidacidal activity of histatin 5

Studies were performed to verify the candidacidal activity of histatin 5 at the different time points 0 min, 30 min, or 60 min. *C. albicans* cell viability after these three incubation time points was determined by plating an aliquot of the cell suspension and comparison of colony forming units (CFUs) in the histatin treated and control samples. Data from the treated samples are shown in Figure 1. For the cells used in the mTRAQ labeling study, on average 100.2% ± 12.4%, 58.3% ± 8.6% and 29.7% ± 9.6% cell survival was observed after 0, 30 and 60 min incubation with histatin 5, respectively. For the cells used in the ICAT labeling study, on average of 105.5% ± 2.1%, 69.3% ± 6.4%, and 40.8% ± 1.4% cell survival was observed after 0, 30 and 60 min, respectively. The survival rates of cells used in the mTRAQ and ICAT labeling studies were not significantly different (t0; p=0.50, t30; p=0.08, t60; p=0.08). As anticipated, the data show that the candidacidal activity of histatin 5 towards *C albicans* is time dependent reaching 50% cell killing between 30 and 60 min of incubation. These samples were used to investigate if the candidacidal cascade was reflected in alterations of protein expression at the mitochondrial level.

Number of identified proteins using mTRAQ and ICAT labeling strategies

In search of protein expression differences, we employed mass spectrometric technologies for protein quantitation using mTRAQ (light and heavy) and ICAT (light and heavy) labeling reagents. For mTRAQ, three biological experiments were carried and for ICAT one experiment. Each experiment was conducted in triplicate. The number of proteins identified by mTRAQ and ICAT at each of the three histatin incubtation time points is shown in Table 1. In the t=0 samples, 45 (mTRAQ) and 17 (ICAT) proteins were identified; in the 30 min samples, 58 (mTRAQ) and 17 (ICAT) proteins were identified and in the 60 min samples, 71 (mTRAQ) and 21 (ICAT) proteins were found. Overall, in all mTRAQ and ICAT analyses combined, 144 different proteins were identified for which protein expression ratios could be determined. Of those, 105 were found by the mTRAQ labeling strategy, 57 by the ICAT labeling strategy and 18 were found by both approaches. The results indicate that the two methods are highly complementary and expand the scope of the number of identified proteins. Almost all proteins found were of mitochondrial origin indicating negligible contamination of the mitochondrial samples with proteins originating from cellar compartments.

Identification and relative quantitation of differentially expressed *C. albicans* mitochondrial proteins upon histatin treatment

Relative quantitations of proteins in control and histatin-treated samples were expressed in ratios and statistical analysis was conducted to establish significant differences. For a subset of the 144 proteins, 6 of 105 proteins in mTRAQ and 26 of 57 proteins in ICAT, insufficient numbers of peptides were identified for statistical analysis and these proteins were excluded. As anticipated, at t=0 min, no differences in protein expression levels between histatin treated and control cells were noted in all 45 (mTRAQ) and 17 (ICAT) proteins identified. In contrast, in the t=30 min and t=60 min samples, significant differences in protein expression were noted. Supplemental Table 1 summarizes all proteins that were identified and their relative expression ratios. Examples of the calculation of the average ratio of peptides identified in the t=0 sample analyzed by mTRAQ is shown in supplemental Table 2. We grouped the proteins in the histatin 5 treated and control cells according to their biological/functional categories (Figure 2). From this analysis it can be observed that most of the proteins that were found to be down-regulated after 30 min and 60 min incubation participate in cellular processes related to cell metabolism and energy conservation. On the other hand, the up regulated proteins were mostly found to function in genome maintenance and cell expression. Specifically, many proteins found in the respiratory complex group and the Krebs cycle were significantly down-regulated. Examples of proteins that were down-regulated at both the 30 min and 60 min time point were ubiquinol cyt-c reductase core protein 2, ATP synthase gamma chain, and malate dehydrogenase. Proteins that were up-regulated were frequently found to be involved in cell rescue or signaling, for instance elongation factor 3, elongation factor 1-alpha and various histones. These apparent groupings were of interest and further validated by Western Blotting.

AtpC and EEF1A1 protein expression

To validate the differential expression levels of proteins identified by mass spectrometry, Western blotting was carried out. A large number of proteins were interesting candidates for validation. We were particularly interested in proteins in the ATP synthase complex, histones and elongation factors, since multiple proteins in these multi-protein complexes showed differential expression. Unfortunately, antibodies were not available for all proteins of interest. In some cases, antibodies were available against the human protein equivalent, but the antibody would not cross react with the protein in *C. albicans* (data not shown). We were able to obtain three primary antibodies against proteins of interest that did recognize *C. albicans* proteins. These were antibodies against *A. thaliana* and *C. reinhardtii* chloroplast-localized gamma chain of ATPase (AptC), against human elongation factor 1 alpha 1 (EEF1A1) and against pigeon cytochrome c (Cytc). Control and histatin treated samples (t=0, 30 min and 60 min) were immunoblotted with these three antibodies. Previous studies have shown that cytochrome c is not released from mitochondria after histatin treatment41 and we also found that cytochrome c shows identical expression levels in the control and experimental group (supplemental Table 1). The Western blotting results obtained are represented in Figure 3. Densitometric analysis from at least two experiments confirms quantitative MS data that AtpC in histatin 5 treated mitochondria is significantly decreased as compared to the control. Data obtained with EEF1A1 also confirm that this protein is up-regulated in response to histatin treatment. While only a small number of proteins could be verified by Western Blotting, the confirmation obtained lends support to the validity of the relative expression ratios acquired with the quantitative MS approach. Our data provide confidence that the quantitative mTRAQ and ICAT proteomics technologies are viable new strategies to gain insights into a cell's proteomic adaptations in the presence of an antifungal agent.

Discussion

It has been well recognized that cellular survival responses are reflected in changes at the mRNA and proteome level. For instance, *C. albicans* cells incubated with a variety of clinically employed antimycotic agents including ketoconazole, amphotericin B and 5-fluorocytosine have shown drug specific changes in gene expression that are in agreement with their established mechanisms of antifungal action.42 The specificity of the *C. albicans* response to these antimycotic agents provides convincing evidence that the analysis of cellular adaptation processes can provide useful information on the mechanism of action of antifungal agents. While the transcriptome analysis reveals expression patterns of specific genes, it does not provide information on the presence or absence of proteins/peptides which are the actual biological entities responsible for cellular function. The field of quantitative proteomics has been applied to study antibiotic resistance in Staphylococcus aureus43, to compare planktonic and biofilm cultures of *Bacillus cereus*44 and to compare rat mitochondrial proteomes of muscle, heart, and liver.45 In *C. albicans*, few studies exploring mitochondrial protein expression differences by mass spectrometry have been described. The present study describes the adaptive cellular responses to histatin 5 at the protein level using state-of-the-art mass spectrometric technologies.

Our data have identified proteins in the *C. albicans* mitochondrial proteome which are impacted upon histatin treatment. Interestingly, histatin 5 treated cells resulted in significant down-regulation of protein expression levels involved in NAD-linked respiration. For instance, malate dehydrogenase (MDH) was significantly down-regulated. Mitochondrial MDH has key functions in the Krebs cycle, which is of central importance to the of use oxygen as part of cellular respiration. MDH catalyzes the conversion of malate into oxaloacetate using NAD^+. In the malate-aspartate shuttle, an increase in mitochondrial NAD/NADH ratio leads to the production of aspartate from malate through MDH, increasing the metabolic fitness of the cells. Histatin 5 treated cells may be impaired in keeping a balance in the mitochondrial NAD/NADH ratio, due to the lower expression of MDH. A recent study has shown that MDH is related to the extension of life span because mitochondrial components of the malate-aspartate shuttle are conserved longevity factors.46 Reduced expression of MDH caused by histatin 5 treatment may thus lead not only to an imbalance of the mitochondrial NAD/NADH ratio in the Krebs cycle, but also to a diminution of life span. Furthermore, Samokhvalov *et al* have reported that a decrease in activity of all NAD-dependent dehydrogenases, such as MDH, in the Krebs cycle is related to a decrease of oxidation coupled with ATP synthesis.47 Down-regulation in the protein expression levels of MDH by exposure to histatin 5 could thus affect ATP synthesis as well.

The other class of proteins that were found to be down-regulated were proteins involved ATP energy metabolism. Recently it has been reported that in *Leishmania*, histatin 5 causes a decrease of mitochondrial ATP synthesis through inhibition of F1F0-ATPase. Our data showed that in *C. albicans* histatin 5 significantly down-regulated the ATP synthase gamma chain after 30 and 60 min of exposure. The asymmetrical gamma chain of F1F0-ATPase rotates inside the cylinder formed by the six alpha and beta subunits and it compels the beta subunits to undergo structural changes.48,49 Since the expression of ATP synthase gamma chain was decreased in histatin 5 treated mitochondria, this would disrupt the production of ATP. Evidence to support significantly lower total ATP levels in histatin treated cells has been reported.24 In contrast to the suppression of the gamma unit, the expression level of the potential mitochondrial inner membrane ATP/ADP translocator showed no change at 30 min, and an increase after 60 min of histatin exposure. From our results, we postulate that mitochondrial ATP synthesis is reduced as a result of through inhibition of the F1F0-ATPase, and that the reduction in mitochondrial ATP is further accelerated by transportation

of ATP from the mitochondrial matrix to the cytoplasm by increased expression of the mitochondrial inner membrane ATP/ADP translocator.

As mentioned above, mitochondrial proteins involved in energy metabolism were found to be down-regulated by histatin 5 treatment. Classes of proteins sharing up-regulation are involved in protein synthesis, including elongation factor 1α, a cell rescue or signaling protein involved in genome maintenance and gene expression. Elongation factor 1α belongs to a set of proteins that facilitate the events of translational elongation and steps in protein biosynthesis.50-52 The elongation phase in ribosomal protein synthesis is at the core of this process and consists of a series of reactions. Other up-regulated proteins were histones and ribosomal proteins. Mitochondrial histones are the major DNA binding proteins in mitochondria and are necessary for maintenance of the mitochondrial genome in yeast during growth. Mitochondrial ribosomal proteins are encoded by mtDNA. Up-regulation of these proteins involved in the control of gene expression and maintenance of mitochondrial DNA (mtDNA) has been suggested to cause reduced cell growth.53 Since a number of ribosomal and histone proteins were increased in *C. albicans* both at 30 min and 60 min exposure time points with histatin 5, the expression of these proteins can be anticipated to have an impact on cell fitness. Notably, some of these proteins were not identified in the t0 samples and overall, more proteins were identified in the t30 and t60 samples. It is not expected that proteins are detected in all samples due to technical limitations inherent to mass spectrometry. This is especially true for those proteins that are present in low abundance. Therefore, the major emphasis for interpretation is on individual protein ratios observed at a particular histatin 5 incubation time point.

By measuring changes in protein expression levels using chemical proteomics strategies, proteomics methods offer an indirect readout of the significant regulated events in protein activity. Regulation of protein expression refers to the control of the amount and timing of appearance of the functional product of a protein. It should be pointed out that protein expression adjustment is one of ways by which cells can adapt to a variable environment. Protein activity can furthermore be regulated by variable substructure, conformation, and post-translational modifications such as phosphorylation. Both regulations of expression and activity help to maintain a stable internal environment in living organisms and control of enzymatic activity could directly influence the protein expression machinery.

We propose a model for the mode of action of the histatin 5 protein taking into account available literature26 as well as the observations reported here. Swelling of mitochondria,55 followed by the dissipation and permeabilization of mitochondrial membrane potential,26 inhibits the activity of the Krebs cycle precipitated by down-regulation of NAD(H)-dependent enzymes. A decrease in ATP production results from the reduced expression of F1F0-ATPase subunits. Lastly upregulation of proteins involved in protein biosynthesis cause reduced cell fitness leading to the release of intracellular nucleotides and other energy storage molecules and cell death.24›25 This model is depicted in Figure 4. Furthermore, these mechanistic pathways can be extrapolated to the *in vivo* condition. Histatin 5 is continually being replenished in the oral cavity by the major salivary glands. Upon contact with whole saliva, histatins have been shown to be cleaved into fragments, many of which retain their antifungal properties 56 and likely act mechanistically similar to histatin 5.

In conclusion, comparative *C.albicans* mitochondrial proteomic analyses in the protein differential expression level between histatin 5 treated and control cells provided a global approach for identifying potential antifungal factors and mechanistic pathways responsible for cell killing by histatin 5. In addition to previous concepts of candidal demise from contact with histatin 5 emphasizing cellular leakage of nucleotides and other energy storage molecules, the MS technologies utilized show that histatins actually may provoke a

suppression and down-regulation of specific mitochondrial proteins involved in metabolism and up-regulation of biosynthesis proteins. The key steps in the histatin 5 antifungal mechanism involve a bioenergetic collapse of *C. albicans*, caused essentially by a decrease in mitochondrial ATP synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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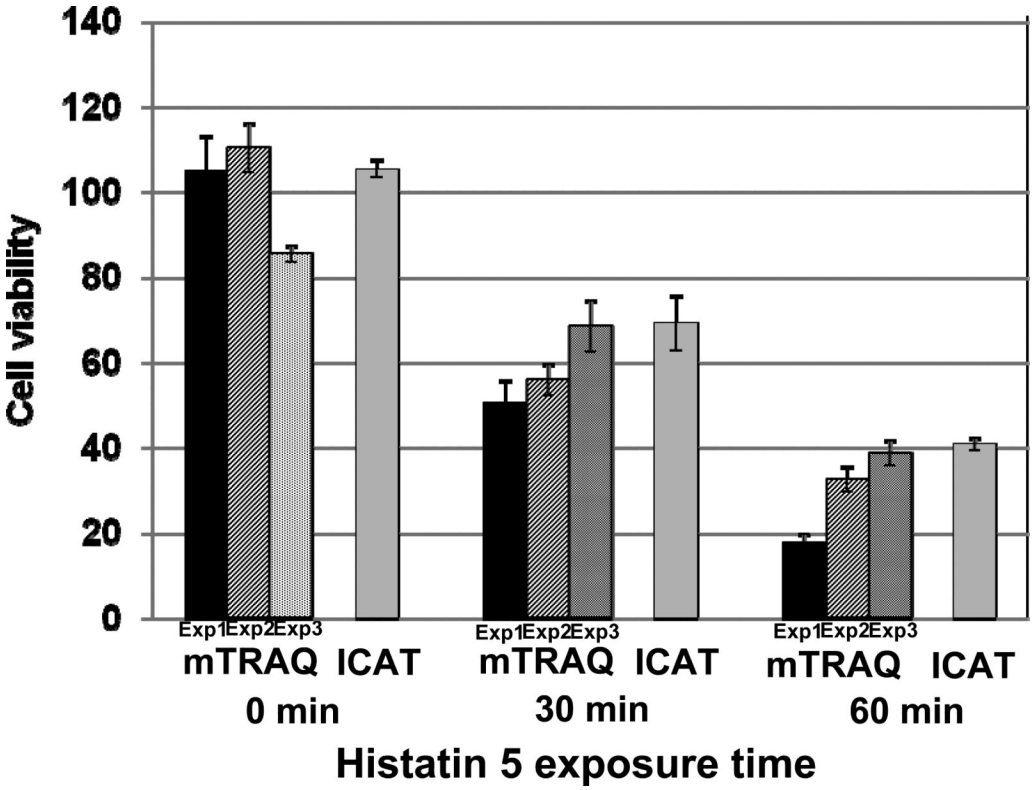

Figure 1.

Viability of *C. albicans* blastoconidia after histatin 5 treatment. Cells were incubated for 0, 30, and 60 minutes with 6 μM of histatin 5 at 37°C and aliquots were plated on agar for viability assessment. Colony-forming units (CFUs) are plotted for three biological experiments in the mTRAQ study (exp1: black bar, exp2: hatched bar, and exp3: dotted bar) and one biological experiment in the ICAT study (gray bar). Data represent the average of CFU/ml in cell aliquots plated in triplicate.

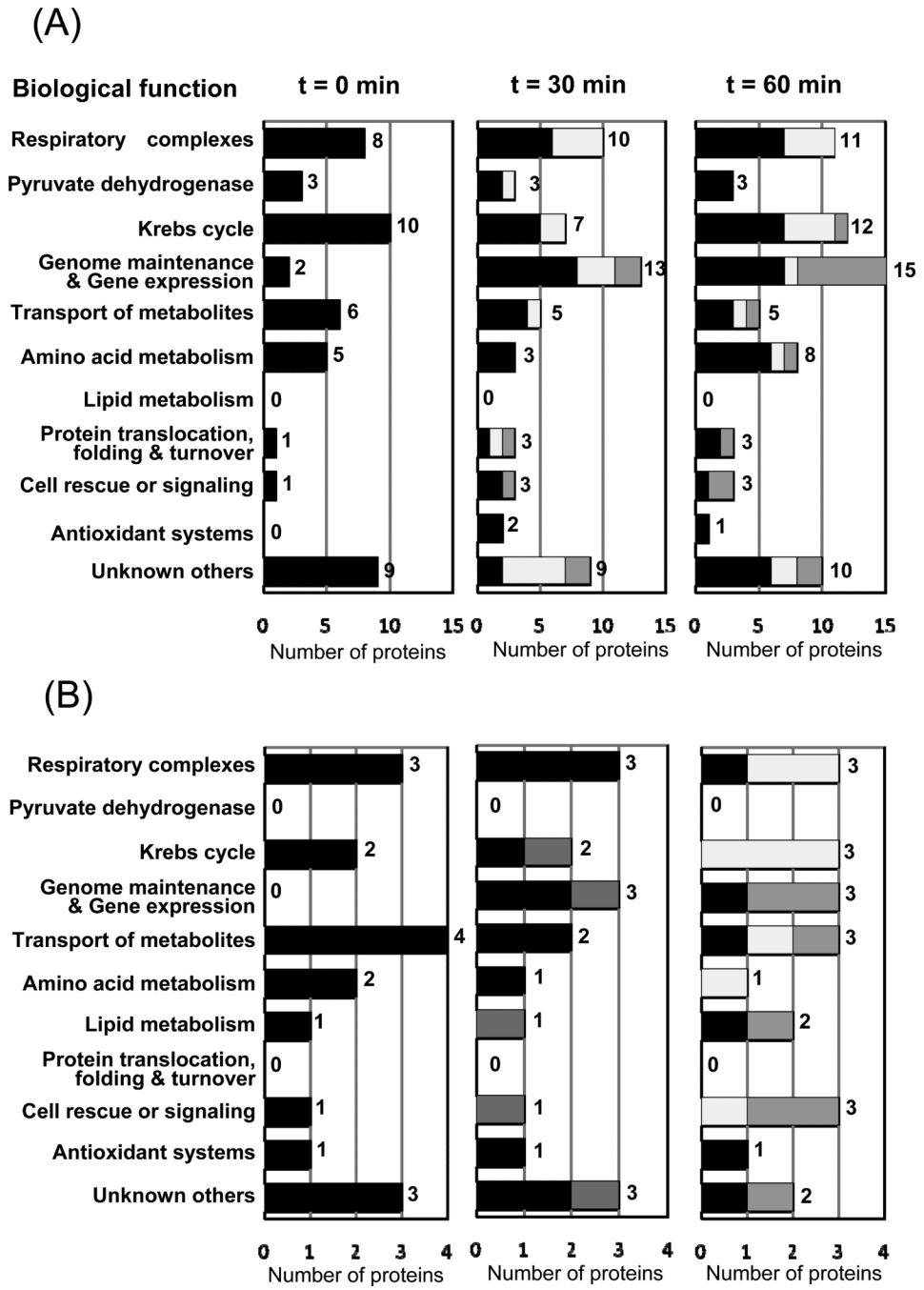

Figure 2.

Distribution of differentially expressed proteins in various mitochondrial biological functional groups. *C. albicans* cells were treated with histatin 5 for 0 min, 30 min or 60 min. Mitochondria were harvested and relative differences in protein expression were assessed with mTRAQ (A) or ICAT (B). Unaffected proteins showing equal levels of expression in the control and treated sample are indicated with black bar segments, down-regulated proteins with white bar segments and up-regulated proteins with gray bar segments. Note that protein expression differences were observed in *C. albicans* incubated for 30 or 60 min with histatin 5, but not in the samples incubated for 0 min.

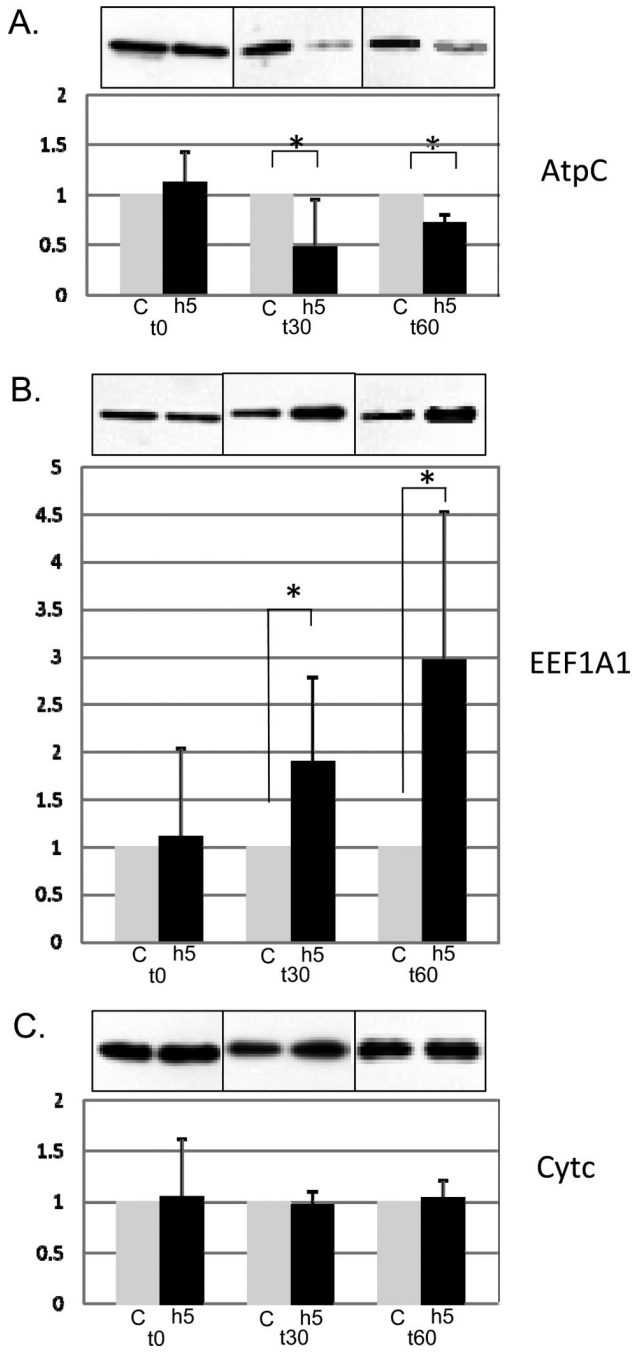

Figure 3.

Western blot analysis of ATP synthase gamma chain and elongation factor 1-alpha 1 proteins in control and histatin-treated mitochondrial samples. Aliquots of 3 μg of mitochondrial proteins were subjected to 12% SDS-PAGE and Western Blotting using specific antibodies against A, the subunit of ATP synthase (AtpC), B, elongation factor 1 alpha 1 (EEF1A1), or C, cytochrome c (Cytc). Representative Western blots are shown as well as the densitometric analyses of experiments conducted at least twice. Data are represented as the mean ± confidence interval (CI) and statistical evaluation for each experimental condition were determined *versus* the control sample. *Experimental value significantly different from the control (p<0.05).

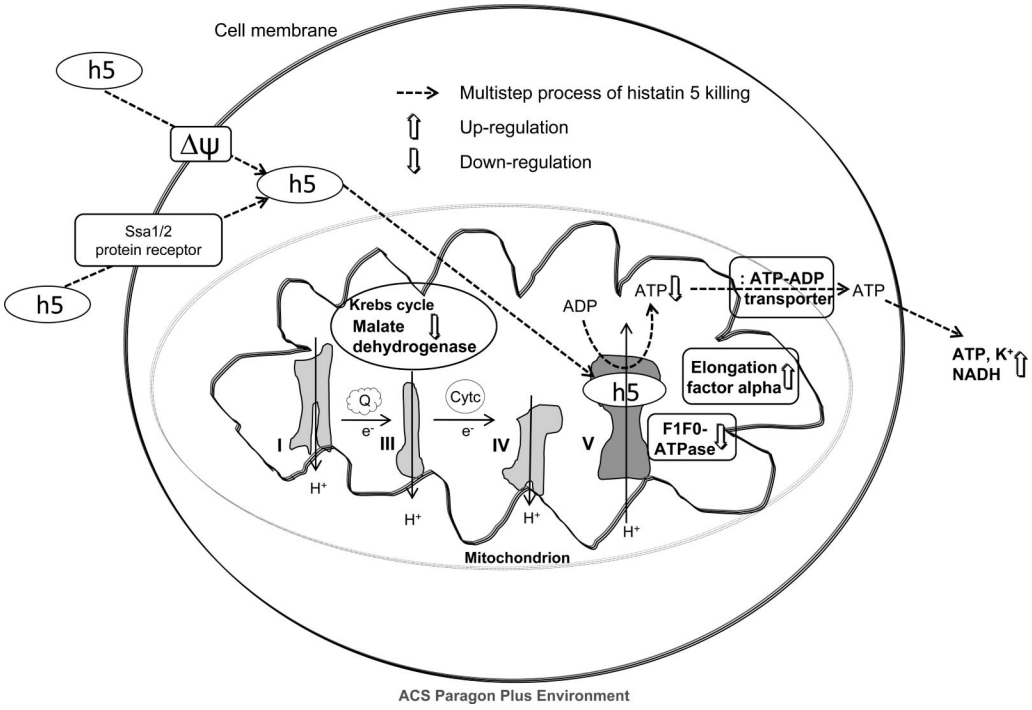

Figure 4.

Hypothetical model for the mode of action of histatin 5 against *C. albicans*. Histatin 5 enters the *C.albicans* cell, involving specific receptors and/or driven by the transmembrane potential. It targets the mitochondria, dissipates the mitochondrial membrane potential, causing mitochondrial swelling. The Krebs cycle is inhibited through down regulation of malate dehydrogenase and the loss of NAD(H) from swollen mitochondria. Histatin reduces the expression of a subunit of the F1F0-ATPase complex, leading to a decrease in ATP production, and ATP levels are further reduced by the apparent upregulation of the ATP-ADP transporter. Upregulation of elongation factor alpha furthermore reduces overall cell fitness. The final result is the release of ATP and other intracellular nucleotides and essential energy storage molecules from the cell and cellular demise.

Table 1

Total proteins identified by mTRAQ and ICAT labeling methodologies and their categorization into up-regulated, down-regulated and unchanged ("same") groups based on expression ratios.

	mTRAQ				ICAT			
	Total	**Up**	**Down**	**Same**	**Total**	**Up**	**Down**	**Same**
t= 0	45	0	0	45	17	0	0	17
t=30	58	6	17	35	17	5	0	12
t=60	71	15	13	43	21	7	8	6