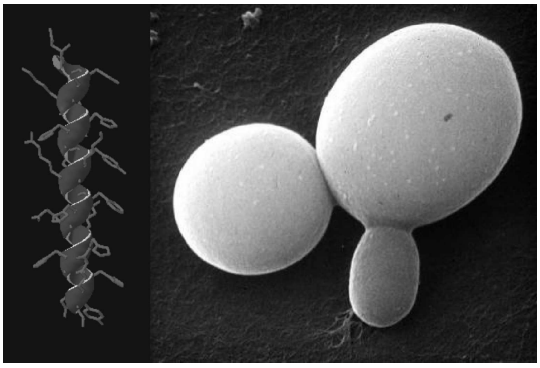

Studie van het celdoodproces in *Candida albicans*

Kris De Smet

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Lijst met afkortingen

A_{260}	absorptie bij 260 nm
ABC	*ATP-binding cassette*
ANOVA	*analysis of variance*
ANT	adenine-nucleotide transporter
ATCC	*American Type Culture Collection*
ATP	adenosine trifosfaat
BH	Bcl-2 homologie
BLAST	*basic local alignment search tool*
bp	baseparen
C. albicans	*Candida albicans*
C. dubliensis	*Candida dubliensis*
C. lusitaniae	*Candida lusitaniae*
C. maltosa	*Candida maltosa*
C. tropicalis	*Candida tropicalis*
C. zeylanoides	*Candida zeylanoides*
cAMP	cyclisch adenosine monofosfaat
CCCP	carbonyl cyanide m-chlorofenylhydrazon
CD	circulair dichroïsme
cDNA	*complementary* DNA
C_t	*threshold cycle*
Da	Dalton
DIDS	*disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate*
$DiOC_5$	*3,3'-Dipentyloxacarbocyanine iodide*
DMSO	dimethylsulfoxide
E. coli	*Escherichia coli*
EDTA	*ethylenediaminetetraacetic acid*
ER	endoplasmatisch reticulum
FACS	*fluorescence activated cell sorting*
FITC	*fluorescein isothiocyanate*
hBD	*human beta-defensin*
HD	*human defensin*
HE	*human epidydimis*
His	histidine
HIV	*human immunodeficiency virus*
HNP	*human neutrophil peptide*
HPLC	*high performance liquid chromatography*
HRP	histidine-rijke proteïnen
Hst	histatine
K. lactis	*Kluyveromyces lactis*
kDa	kilo Dalton
LPS	lipopolysaccharide
Lys	Lysine
MALDI-TOF	*matrix assisted laser desorption ionization time-of-flight*
MD	*molecular dynamics*
mRNA	*messenger* RNA
MRS	*major repeat sequence*
MS	massaspectrometrie
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide fosfaat
NMR	nucleaire magnetische resonantie
OD_{600}	optische densiteit bij 600 nm
ORF	*open reading frame*
P. pastoris	*Pichia pastoris*

Pb	*parotid basic*
PCD	*programmed cell death*
PCR	*polymerase chain reaction*
PKA	proteïne kinase A
PMA	*phorbol 12-myristate 13-acetate*
PPb	*post-parotid basic*
Q-PCR	*quantitative PCR*
rDNA	ribosomaal DNA
ROS	*reactive oxygen species*
ROX	rhodamine-X
rpm	rotaties per minuut
RPS	*repeated sequence*
RT	reverse transcription/real-time
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
MTL	*mating type locus*
S_P	fosfoserine
TFE	2,2,2-trifluoroethanol
TRAIL	*TNF-related apoptosis-inducing ligand*
tRNA	transfer RNA
Tyr	tyrosine
U	unit
UV	ultraviolet
VDAC	*voltage-dependent anion-selective channel*
Y. lipolytica	*Yerwinia lipolytica*
yE*GFP*	*yeast enhanced GFP*

Leidraad

Het voorgestelde werk is opgedeeld in 5 delen.

In de literatuurstudie worden eerst de antimicrobiële peptiden in het algemeen besproken, waarna een uitgebreide literatuurstudie volgt over histatine 5. De histatine-familie, de structuur van histatine 5 en vooral het werkingsmechanisme van histatine 5 worden in detail behandeld. Dit deel bevat één manuscript. Een review artikel geschreven op vraag van Prof. C. Ratledge (editor van *Biotechnology Letters*) dat gepubliceerd werd in *Biotechnology Letters*. Het beschrijft de belangrijkste antimicrobiële peptiden die voorkomen in de mens: de defensines, het cathelicidine LL-37 en de histatines. Verder wordt in de inleiding aandacht besteed aan het organisme dat voornamelijk gebruikt wordt in dit werk, *C. albicans*. En als laatste wordt het pro-apoptotische eiwit Bax en Bax-geïnduceerde celdood in verschillende gisten kort besproken.

In het tweede deel worden de doelstellingen van dit doctoraatswerk omschreven.

Het derde deel omvat de resultaten en is onderverdeeld in 4 hoofdstukken. Hoofdstuk 1 is een manuscript dat gepubliceerd is in *Yeast*. Het beschrijft in detail de expressie van Bax en Gfp-Bax in *C. albicans* en de gevolgen voor de gistcellen. Hoofdstuk 2 beschrijft de synthese en karakterisatie van histatine 5 en de introductie van een celdoodproces in *C. albicans* door middel van incubatie van de cellen met histatine 5. Hoofdstuk 3 bestaat uit een manuscript dat gepubliceerd werd in *Biotechnology Letters* en dat het celdoodproces geïnduceerd door histatine 5 vergelijkt in twee verschillende gisten, *C. albicans* en *S. cerevisiae*, vooral met betrekking tot de activiteit van de mitochondriën. Het laatste hoofdstuk bevat een manuscript dat ingediend werd ter publicatie in het tijdschrift *Eukaryotic Cell*. Het beschrijft de identificatie van genen, die een invloed hebben op het histatine 5-geïnduceerde celdoodproces, door middel van *screening* van een overexpressiebank en door middel van microarray analyse.

In het vierde deel worden de resultaten samengevat.

Het laatste deel (addendum) bevat een publicatie waarvan ik niet de eerste auteur ben. Het manuscript beschrijft de transcriptionele reactie van *S. cerevisiae* op Bax-expressie en deze transcriptionele reactie wordt vergeleken met de transcriptionele reactie op H_2O_2. Verder wordt de invloed van *OYE3* op Bax-geïnduceerd NADPH verlies en lipide peroxidatie besproken.

Deel I
LITERATUURSTUDIE

LITERATUURSTUDIE

In dit eerste deel zullen de elementen besproken worden die de basis vormen van de resultaten die in deze thesis voorgesteld zullen worden. In het eerste hoofdstuk van dit deel zullen de antimicrobiële peptiden besproken worden, waarbij vooral de histatines in detail behandeld zullen worden. Dit eerste hoofdstuk bevat een review die geschreven is op uitnodiging van de editor van *Biotechnology Letters*, Professor Colin Ratledge, en die gepubliceerd is in dit tijdschrift. *Candida albicans,* het organisme dat hoofdzakelijk gebruikt werd als onderzoeksobject in dit werk zal besproken worden in een tweede hoofdstuk. Verder zal in een laatste hoofdstuk Bax-geïnduceerde celdood in gist kort besproken worden.

1 Antimicrobiële peptiden

1.1 Het aangeboren immuunsysteem en antimicrobiële peptiden

De overleving van multicellulaire organismen in een omgeving vol met pathogenen is afhankelijk van een goed werkend immuunsysteem, aangezien ze voortdurend worden blootgesteld aan potentieel schadelijke micro-organismen. Het immuunsysteem bestaat uit het aangeboren, niet-specifiek immuunsysteem en het adaptief immuunsysteem. Het aangeboren immuunsysteem zorgt voor een snelle detectie en vernietiging van schadelijke micro-organismen. De werking van dit aangeboren immuunsysteem vereist, in tegenstelling tot het adaptief immuunsysteem, geen voorafgaande periode van inductie, het hangt immers niet af van de klonale expansie van antigenspecifieke lymfocyten.

Het immuunsysteem kan beschouwd worden als een netwerk van verdedigingsmechanismen, die werkzaam zijn op verschillende niveaus. Het initiële contact van pathogene micro-organismen met de gastheer vindt gewoonlijk plaats ter hoogte van de innerlijke en uiterlijke lichaamsoppervlakken (door contact, inslikken, inademen), zoals de huid en de epitheliale oppervlakken van het gastro-intestinaal, het uro-genitaal en het respiratorisch stelsel. Het contact van de micro-organismen met gastheerweefsel kan verschillende gevolgen hebben:

1) De binnendringende micro-organismen worden geëlimineerd door het aangeboren immuunsysteem van de gastheer zonder een inflammatoire reactie of de activatie van het adaptief immuunsysteem.
2) De initiële werking van het aangeboren immuunsysteem is niet voldoende om het micro-organisme te bestrijden. Als gevolg hiervan worden de effector mechanismen van het aangeboren immuunsysteem opgereguleerd. Deze effectoren hebben directe antimicrobiële activiteit en recruteren inflammatoire cellen en cellen van het adaptief immuunsysteem wat uiteindelijk resulteert in de eliminatie van het micro-organisme. In

dit scenario houdt het aangeboren immuunsysteem de verdubbelingstijd van het micro-organisme voldoende onder controle om een overstelping van het immuunsysteem te vermijden.

3) Het micro-organisme groeit het aangeboren en adaptief immuunsysteem boven het hoofd. Deze situatie leidt, gepaard gaande met een sterke inflammatoire respons, tot de dood van de gastheer.
4) Micro-organismen met specifieke fysiologische aanpassingen kunnen lange tijd overleven in de gastheer. In dit geval zijn de acties van het immuunsysteem niet voldoende om de indringer te verwijderen (Bals, 2000).

Het aangeboren immuunsysteem is de eerste-lijnsverdediging van een gastheer tegen infectie. Het is de eerste barrière die micro-organismen moeten passeren om de gastheer binnen te dringen. Deze barrière is opgebouwd uit veschillende componenten zoals de epitheliale barrière, slijmproductie, beweging van cilia, lage pH van het gastro-intestinaal stelsel, het uro-genitaal stelsel en van de huid, de aanwezigheid van fagocyterende cellen, niet-specifieke humorale factoren, alternatief geactiveerde complement cascade, enzoverder (Kamysz *et al.*, 2003). Het is in staat om onderscheid te maken tussen *self* en *non-self* door de herkenning van geconserveerde microbiële structuren. Het resultaat is de vernietiging en verwijdering van de microbiële indringers en de regulatie van de inflammatoire respons (Ryley, 2001).

Indien de epitheliale barrière van het lichaam is doorbroken, bezit het aangeboren immuunsysteem over een waaier reeds aanwezige, snel mobiliseerbare cellen zoals neutrofielen en macrofagen, epitheelcellen, mastcellen, eosinofielen, en *natural killer* cellen om de binnendringende micro-organismen initieel te bestrijden. Deze cellen expresseren een grote variëteit aan receptoren, zoals *toll-like* receptoren en C-lectine receptoren die geactiveerd worden door componenten van de microbiële pathogenen. Dit resulteert in de vrijstelling en/of activatie van meerdere effectormoleculen en mediatoren van het gastheer immuunsysteem zoals de complement cascade, cytokines, chemokines, superoxides, stikstofoxides, prostaglandines, acute fase proteïnen en antimicrobiële peptiden (Oppenheim *et al.*, 2003).

Antimicrobïele peptiden zijn wijd verspreid in de natuur. Ze werden geïsoleerd uit verschillende organismen waaronder bacteriën, schimmels, planten, invertebraten en vertebraten. De eerste raporten van plantpeptiden met antibacteriële of antifungale werking dateren van de vroege jaren zeventig (Fernandez de Caleya *et al.*, 1972). De laatste vijfentwintig jaar zijn een grote verscheidenheid aan antimicrobiële peptiden ontdekt. Het aantal antimicrobiële peptiden van dierlijke en plantaardige oorsprong is gestegen tot verschillende honderden en databanken zijn voorhanden (http://aps.unmc.edu/AP/main.html, http://www.bbcm.univ.trieste.it/~tossi/pag1.htm en http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/) (Brahmachary *et al.*, 2004; Wang & Wang, 2004).

Door de groeiende resistentie van bacteria en fungi voor de algemeen gebruikte antibiotica en door het besef dat antimicrobiële peptiden en synthetische analogen hiervan potentieel gebruikt kunnen worden als therapeutica, is er een groeiende interesse in deze peptiden (Reddy *et al.*, 2004). Verder hebben de meeste van deze peptiden een brede waaier van eigenschappen. Naast hun antimicrobiële activiteit kunnen ze verschillende biologische processen beïnvloeden zoals inflammatie, proliferatie, wondheling, vrijstelling van cytokines, homeostasis, chemotaxis en het behoud van het evenwicht tussen proteasen en protease inhibitoren (Bals, 2000). De antimicrobiële peptiden verschillen sterk in aminozuur samenstelling, lengte en secundaire structuur. Ondanks deze verschillen spelen ze allemaal een essentiële rol in het immuunsysteem door het verhinderen of limiteren van infecties gebaseerd op hun capaciteit om specifiek potentiële pathogenen te herkennen.

Door de grote variëteit aan antimicrobiële polypeptiden is de nood gerezen om deze in verschillende groepen onder te brengen. Omdat ook antibacteriële en antifungale proteïnen deel uitmaken van het aangeboren immuunsysteem, zoals lysozyme, cathepsine G en *bacteria permeability increasing protein* (BPI) (Levy, 1996), moet er vooreerst een onderscheid gemaakt worden tussen proteïnen en peptiden. Een algemene definitie zegt dat polypeptiden met een moleculair gewicht groter dan 10 kDa proteïnen worden genoemd en polypeptiden kleiner dan 10 kDa, peptiden. Soms kan het onderscheid tussen proteïne en peptide nog artificiëler zijn omdat het gecodeerde genproduct, een precursor (zoals bij de cathelicidinen en lactoferrine), gedefineerd wordt als proteïne, terwijl de eindproducten peptiden zijn (PR39 en lactoferricine, respectievelijk).

De eerste classificatie van antimicrobiële peptiden op een taxonomische basis bleek niet te voldoen door het feit dat dezelfde structurele patronen voorkomen in peptiden van totaal verschillende organismen. Daarom werd een alternatieve classificatie voorgesteld (Tabel 1). Op basis van hun algemene aminozuursamenstelling en secundaire structuur kunnen antimicrobiële peptiden ingedeeld worden in drie klassen (Andreu & Rivas, 1998; Boman, 1998; Bals, 2000).

1) Lineaire, α-helicale peptiden zonder cysteïnes. Tot deze groep behoren bijvoorbeeld de cecropines van het varken, magainines en dermaseptines van de kikker en LL-37/hCAP-18 van de mens.
2) Peptiden met een even aantal cysteïnes, die altijd intramoleculair gebonden zijn. De peptiden kunnen één (thanatin, *bovine* dodecapeptide), twee (protegrines), drie (defensines) of meer (drosomycine van de fruitvlieg) zwavelbruggen bevatten.
3) Lineaire peptiden rijk aan één of twee aminozuren; zoals proline, arginine, tryptofaan of histidine. Deze peptiden bevatten meestal geen cysteïne. Voorbeelden zijn de apidaecines van de bij, bactenicine 5 en bactenicine 7 van rund, PR-39 van varken, histatines, … .

De belangrijkste antimicrobiële peptiden die bij mensen zijn geïdentificeerd, behoren tot de defensines, cathelicidines en histatines.

Groep 1: Lineaire, α-helicale peptiden zonder cysteïnes		
Peptide	Sequentie	Organisme
LL-37:hCAP18	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	mens (*Homo sapiens*)
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIAIQGGPR	varken (*Sus scrofa*)
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	kikker (*Xenopus laevis*)
Groep 2: Peptiden met cysteïnes verbonden door zwavelbruggen		
Peptide	Sequentie	Organisme
Eén zwavelbrug		
Bovine dodecapeptide	RLCRIVVIRVCR	rund (*Bos taurus)*
Thanatin	GSKKPVPIIYC-NRRTGKCQRM	insekt *(Podiscus maculiventris*)
Twee zwavelbruggen		
Protegrin I	RGGRLCYCRRRFCVCVGR	varken (*Sus scrofa*)
Drie zwavelbruggen (defensines)		
α-defensine familie		
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	mens (*Homo sapiens*)
HD-5	ARATCYCRTGRCATRESLSGVCEISGRLYRLCCR	mens (*Homo sapiens*)
RK-1	MPCSC-KKYCDPWEVIDGSC-GLFNSKYICCREK	konijn (*Oryctolagus cuniculus*)
β-defensine familie		
hBD-1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK	mens (*Homo sapiens*)
TAP	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK	rund (*Bos taurus)*
Gallinacin I	GRKSDCFRKSGFCAFLKCPSLTLISGKC-SRFYLCCKRIW	kip (*Gallus gallus*)
θ-defensine familie (cyclische peptiden)		
rTD-1	GFCRCLCRRGVCRCICTR	rhesus aap (M*acaca mulatta*)
Insect defensines		
Sapecin	LTCEIDRSLCLLHCRLKGYLRAYCSQQKVCRCVQ	vlieg (*Sarcophaga peregrina)*
Meer dan drie zwavelbruggen		
Drocomycin	DCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCSPSLKCWCEGC	fruitvlieg (*Drosophila melanogaster)*
Groep 3: Lineaire peptiden rijk aan bepaalde aminozuren		
Peptide	Sequentie	Organisme
Abaecin	YVPLPNVPQPGRRPFPTFPGQGPFNPKIKWPQGY	bij (*Apis mellifera*)
Bactenecin 5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLRFP	rund (*Bos taurus)*
Histatin 1	DSHEKRHHGYRRKFHEKHHSHKEFPFYGDYGSNYLYDN	mens (*Homo sapiens*)
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	varken (*Sus scrofa*)

Tabel 1. Algemene classificatie van antimicrobiële peptiden. Cysteïne residuen in de defensines zijn onderlijnd.

1.2 Antimicrobiële peptiden van de mens

Naar aanleiding van de publicatie van het artikel '*Role of oxidative phosphorylation in histatin 5-induced cell death in* Saccharomyces cerevisiae' in het tijdschrift *Biotechnology Letters*, werden we uitgenodigd door Professor Colin Ratledge, editor van dit tijdschrift, om een mini-review te schrijven over antimicrobiële peptiden. Het resultaat is een review die de belangrijkste menselijke antimicrobiële peptiden behandelt: de defensines, het cathelicidine LL-37 en de histatines. In deze review worden in het kort de structuur en de activiteit van deze peptiden besproken. Verder wordt het werkingsmechanisme van histatine 5 besproken en vergeleken met het werkingsmechanisme van defensines en LL-37.

Review artikel

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Human antimicrobial peptides: defensins, cathelicidins and histatins.
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Human antimicrobial peptides: defensins, cathelicidins and histatins

Kris De Smet[1,2] & Roland Contreras[1,]*

[1]Unit of Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, B-9052 Ghent, Belgium
[2] Current address : Flen Pharma NV, Drie Eikenstraat 661, B-2650 Edegem, Belgium.

*Author for correspondence (Fax: ++32 9 3313502 ; E-mail: Roland.Contreras@dmbr.UGent.be)

Key words: antimicrobial peptides, cathelicidin, defensins, histatins, innate immunity

Abstract

Antimicrobial peptides, which have been isolated from many bacteria, fungi, plants, invertebrates and vertebrates, are an important component of the natural defenses of most living organisms. The isolated peptides are very heterogeneous in length, sequence and structure, but most of them are small, cationic and amphipathic. These peptides exhibit broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, fungi and enveloped viruses. A wide variety of human proteins and peptides also have antimicrobial activity and play important roles in innate immunity. In this review we will discuss three important groups of human antimicrobial peptides. The defensins are cationic nonglycosylated peptides containing six cysteine residues that form three intramolecular disulfide bridges resulting in a triple-stranded β-sheet structure. In humans, two classes of defensins can be found: α-defensins and β-defensins. The defensin-related HE2 isoforms will also be discussed. The second group is the family of histatins, which are small, cationic, histidine-rich peptides present in human saliva. Histatins adopt a random coil structure in aqueous solvents and form α-helices in non-aqueous solvents. The third group comprises only one antimicrobial peptide, the cathelicidin LL-37. This peptide is derived proteolytically from the *C*-terminal end of the human CAP18 protein. Just like the histatins, it adopts a largely random coil conformation in a hydrophilic environment, and a α-helical structure in a hydrophobic environment.

Introduction

Multicellular organisms are permanently exposed to thousands of potentially pathogenic microorganisms. Innate immunity forms a first line of defense against infection by these microorganisms. An important part of innate immunity is a group of peptides with antimicrobial activity. Initial reports of plant peptides with antibacterial or antifungal properties date from the early 1970s (Fernandez de Caleya *et al.*, 1972). Over the past twenty years, a great diversity of peptide antibiotics has been discovered. Antimicrobial peptides from plants and animals now number in the hundreds, and databases have been established (http://aps.unmc.edu/AP/main.html, http://www.bbcm.univ.trieste.it/~tossi/pag1.htm and http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/) (Brahmachary *et al.*, 2004; Wang & Wang, 2004). In view of the increasing resistance of bacteria and fungi to the commonly used antibiotics, there is growing interest in peptide antibiotics, driven by awareness of the potential therapeutic applications of these peptides or their synthetic analogues (Reddy *et al.*, 2004). All these peptides have a broad range of biological properties. Besides antibacterial and antifungal activities, some of these peptides also possess antiviral or anticancer properties. Furthermore, they can influence inflammation, proliferation, wound healing, release of cytokines, homeostasis, chemotaxis and the preservation of a balance between proteases and protease inhibitors (Bals, 2000). These peptide antibiotics differ widely in their biochemical properties (amino acid composition, length and secondary structure). However, they all play essential roles in nonspecific host defenses by preventing or limiting infections by their ability to selectively recognize potential pathogens. Most peptides exert their antifungal or antibacterial effects by interacting with and destabilizing the microbial membrane, leading to cell death. However, different modes of action are proposed for several peptides, including inhibiting synthesis of specific membrane proteins (Engstrom *et al.*, 1984; Axen *et al.*, 1997) or stress proteins (Groisman, 1996), arrest of DNA synthesis (Boman *et al.*, 1993), breakage of single-strand DNA (Bateman *et al.*, 1991), interaction with DNA (Park *et al.*, 1998), and production of hydrogen peroxide (Leem *et al.*, 1996). Antimicrobial peptides can also act by triggering apoptosis in eukaryotic cells (Velasco *et al.*, 1997; Yoo *et al.*, 1997) or autolysis in bacterial targets (Chitnis *et al.*, 1993).

Human antimicrobial peptides

A wide variety of human proteins and peptides have antimicrobial activity. This review will be limited to discussing three important groups: defensins (and the related HE2 isoforms), the cathelicidin LL-37 and histatins.

Defensins

Mammalian defensins are cationic nonglycosylated peptides with arginine as the primary cationic residue. They have molecular masses of 3.5 – 6 kDa and contain six cysteine residues that form three intramolecular disulfide bridges (Lehrer, 2004; Ganz, 2005). In humans, two classes of defensins can be found: α-defensins and β-defensins (Table 2).

The α-defensins are 29–35 amino acids long; the three disulfide bridges are between residues 1 and 6, 2 and 4, and 3 and 5, resulting in peptides forming a triple-stranded β-sheet structure with a β-hairpin loop containing cationic charged molecules (Fig. 1A). In humans, four α-defensins have been isolated from neutrophils (HNP-1 to 4). All four α-defensins can be found in the azurophilic granules of neutrophil granulocytes. Half of the azurophilic protein content is composed of HNP-1, 2 and 3, whereas HNP-4 is present at lower concentrations (Ganz *et al.*, 1990). HNP-1 to 3 are also found in B cells and natural killer cells. In neutrophils, the α-defensins play a role in the oxygen-independent killing of phagocytosed microorganisms. Two α-defensins (HD-5 and 6) are referred to as enteric defensins, and are found in the granules of Paneth cells of the small intestine and in the epithelial cells of the female urogenital tract (Jones & Bevins, 1992, 1993). The genes for all six α-defensins are found in the same region of chromosome 8. Alpha-defensins are expressed as prepropeptides that have no antimicrobial activity. The *C*-terminal part of the peptide is responsible for the antimicrobial activity. In the case of the enteric defensins, a single metalloproteinase is responsible for the release of the active peptide.

Human β-defensins are somewhat larger than α-defensins. Although there is little primary sequence homology between these two defensin families, their tertiary structures are very similar because of the presence of three disulfide bonds. In β-defensins the three disulfide bridges are between residues 1 and 5, 2 and 4, and 3 and 6, also resulting in peptides with a triple-stranded β-sheet structure and a β-hairpin loop containing cationic charged molecules (Lehrer, 2004) (Fig 1B). The first human β-defensin (hBD-1) was isolated from hemofiltrate of patients undergoing dialysis treatment. hBD-1 is expressed in epithelia that are directly exposed to the environment or microbial flora (e.g. in the lung, mammary gland, salivary gland, kidney, pancreas and prostate) (Bensch *et al.*, 1995). A second member of the family, hBD-2, was first characterized in psoriatic skin. hBD-2 is widely expressed in epithelia (lung, gut, urogenital

system, pancreas and skin), leukocytes and the bone marrow. In contrast to hBD-1, exposure of epithelial tissue to LPS or pro-inflammatory agents (TNF-α or IL-1β) upregulates the expression of hBD-2 (Harder *et al.*, 1997). Another defensin (hBD-3) was isolated from human lesional psoriatic scales and cloned from keratinocytes. hBD-1 and 2 show microbicidal acitivity predominantly against Gram-negative bacteria, and only low, if any, microbicidal activity against Gram-positive bacteria. hBD-3 is a broad spectrum peptide antibiotic that kills many potential pathogenic bacteria and the opportunistic pathogenic yeast, *Candida albicans* (Table 3). Like hBD-2, hBD-3 is also induced by inflammatory stimuli, such as TNF-α and contact with bacteria. Skin and tonsils were found to be the major tissues expressing the hBD-3 mRNA (Harder *et al.*, 2001). These first three human β-defensins were discovered via the identification of antimicrobial substances in large amounts of biological material. The genes of these three β-defensins are located in a single cluster at chromosomal region 8p23. Using the basic local alignment search tool (BLAST), three other β-defensin genes were discovered in this region (hBD4-6) (Garcia *et al.*, 2001; Yamaguchi *et al.*, 2002). hBD-4 was found to be highly expressed in the testis. However, Yamaguchi *et al.* (2002) could not confirm this, but they showed that hBD-4, hBD-5 and hBD-6 are expressed in the human epididymis. The low basal expression of hBD-4 in lung epithelial cells could be upregulated by contact with bacteria or by phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C. However, expression of hBD-4 was not induced by exposure to IL-1α, IL-6, IFNγ or TNF-α. hBD-4 inhibited the growth of Gram-positive and Gram-negative bacteria and of the yeast, *Saccharomyces cerevisiae* (Garcia *et al.*, 2001). Yamaguchi *et al.* (2002) proposed the division of the β-defensins in two groups: the epididymis-specific isoforms and the other isoforms. The epididymis-specific isoforms include hBD-4, hBD-5, and hBD-6. The other isoforms include hBD-1, hBD-2 and hBD-3 (Yamaguchi *et al.*, 2002). All of these defensin genes are located in a single gene cluster at chromosomal region 8p23. Using a computational search tool based on hidden Markov models in combination with BLAST, 3 new β-defensin gene clusters and 28 new human β-defensin genes were discovered (Schutte *et al.*, 2002). Human β-defensin 118 is a recently characterized epididymis-specific peptide that binds spermatozoa and has potent antimicrobial activity (Yenugu *et al.*, 2004a).

Besides their antimicrobial activity, defensins also show additional properties (Kamysz *et al.*, 2003; Oppenheim *et al.*, 2003), such as antitumor activity (Lichtenstein *et al.*, 1986), stimulation of cell proliferation (Murphy *et al.*, 1993), interference with signal transduction pathways (Charp *et al.*, 1988), chemoattraction of immune cells (Territo *et al.*, 1989), and stimulation of cytokine and adhesion molecule expression (Chaly *et al.*, 2000).

Human epididymis 2 (HE2) protein isoforms

HE-2, a gene expressed in human epididymis (Osterhoff *et al.*, 1994) gives rise to multiple mRNAs that encode a family of small cationic secretory peptides of 4 to 8 kDa. The localization of the HE-2 gene in the defensin gene cluster on chromosome 8 and homology to the antimicrobial β-defensins suggest that these peptides play a role in the innate epithelial defense system of the epididymal duct. The HE-2 isoforms contain identical proregions joined to different *C*-terminal peptides (Hamil *et al.*, 2000). The *C*-terminal peptides are cleaved from their proregions by a furin-like proprotein convertase, just like the β-defensins. HE-2α and HE-2β1 are the most prevalent isoforms. The β-defensin-like HE-2β1 isoform has the expected antimicrobial activity. But the HE-2α isoform also performs antimicrobial activity, although this peptide does not share significant similarity with β-defensins (von Horsten *et al.*, 2002; Yenugu *et al.*, 2004b).

Cathelicidins

Cathelicidins are a family of antimicrobial peptides derived from proteins, that contain a highly conserved signal sequence and a proregion highly homologous to cathelin, a cathepsin L inhibitor, but the cathelicidin *C*-terminal domain shows substantial heterogeneity (Hancock & Diamond, 2000; Sorensen & Borregaard, 2005). In humans only one cathelicidin has been characterized, LL-37 (Table 2). This peptide is derived by proteolysis from the *C*-terminal end of the human CAP18 protein (hCAP18) (Gudmundsson *et al.*, 1996) and is expressed in leukocytes such as neutrophils, monocytes, NK cells, T cells and B cells, and in epithelial cells of the testis, skin, and the gastrointestinal and respiratory tracts (Cowland *et al.*, 1995; Gudmundsson *et al.*, 1996; Frohm *et al.*, 1997; Bals *et al.*, 1998; Agerberth *et al.*, 2000). LL-37 is induced by inflammatory or infectious stimuli (Frohm *et al.*, 1997) and has antimicrobial activity against both Gram-positive and Gram-negative bacteria (Turner *et al.*, 1998) (Table 3). Besides its antimicrobial activity, the peptide binds and neutralizes LPS and protects against endotoxic shock in a murine model of septicemia (Bals *et al.*, 1999). Furthermore, it is chemotactic for neutrophils, monocytes, mast cells and T cells, induces degranulation of mast cells, alters transcriptional responses in macrophages, stimulates wound vascularization and re-epithelialization of healing skin (Zanetti, 2004), and has antitumor activity (Okumura *et al.*, 2004). LL-37 is composed of 37 amino acid residues, and has a linear structure because it does not contain cysteine. The peptide adopts a largely random coil conformation in a hydrophilic environment, and an α-helical structure in a hydrophobic environment (Turner *et al.*, 1998).

Histatins

Histatins comprise a family of small, cationic, histidine-rich peptides of 3-4 kDa present in human saliva (MacKay *et al.*, 1984a). The peptides are constitutively produced and secreted by the submandibular, sublingual and parotid glands (vanderSpek *et al.*, 1989; Ahmad *et al.*, 2004). These histidine-rich peptides were first described in the early 1970s as peptides that enhance the glycolytic activity of microorganisms (Holbrook & Molan, 1973). Later reports described their potent bactericidal (MacKay *et al.*, 1984b) and, more importantly, fungicidal properties (Pollock *et al.*, 1984) (Table 3). These peptides form part of the innate immune system and play an important role in maintaining oral health by limiting infections in the oral cavity. The histatin family consists of several members (Castagnola *et al.*, 2004), of which histatin 1, 3 and 5 are the most important (Table 2). The histatins are encoded by two closely related genes (*HIS1* and *HIS2*), with histatin 1 and histatin 3 as primary products of *HIS1* and *HIS2*, respectively. Histatin 5 is formed by further processing of histatin 3 (Sabatini & Azen, 1989). Characterization of these three peptides showed that they have linear structures containing 38, 32 and 24 amino acid residues, respectively, and that each of them has seven histidine residues (Oppenheim *et al.*, 1988). Characterization of the secondary structure revealed that histatin 5 adopts a random coil structure in aqueous solvents and an α-helix structure in non-aqueous solvents (Fig. 2) (Raj *et al.*, 1998). Of all histatins, histatin 5 has the strongest antimicrobial activity, and most of the research on histatins has focused on this peptide. It has potent antifungal activity against the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Tsai & Bobek, 1997; Helmerhorst *et al.*, 1999a). Besides their antimicrobial activity, histatins also possess other properties: they bind to hydroxyapatite (Jensen *et al.*, 1992), human salivary mucin MG1 (Iontcheva *et al.*, 1997), and tannins, plant-derived polyphenolic compounds (Yan & Bennick, 1995). Furthermore, histatin 5 inhibits inflammatory cytokine induction from human fibroblasts (Imatani *et al.*, 2000), inhibits the leukotoxin activity of *Actinobacillus actinomycetemcomitans* (Murakami *et al.*, 2002), inhibits host and bacterial enzymes implicated in periodontal disease (Gusman *et al.*, 2001a), and exhibits metallopeptide-like properties (Gusman *et al.*, 2001b).

P-113, a 12-amino-acid fragment of histatin 5, was identified as the smallest fragment that retains anticandidal activity comparable to that of the parent compound (Rothstein *et al.*, 2001). This peptide was used in a mouth rinse and gel formulations in phase I/II clinical trials to evaluate its safety and its efficacy against plaque and gingivitis. The data of both studies indicated that these formulations are safe and tolerated by humans. In addition, they reduce the development of gingival bleeding, gingivitis and plaque in a human experimental gingivitis model (Paquette *et al.*, 2002; Van Dyke *et al.*, 2002).

Mode of action

Antimicrobial peptides such as defensins, cathelicidins and histatins have broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, fungi and enveloped viruses. The general mode of action of the defensins and cathelicidin is electrostatic binding of the cationic peptide to the outer surface of the pathogen, followed by insertion of the peptide into the cytoplasmic membrane, resulting in leakage of the cell contents into the extracellular medium (White *et al.*, 1995; Bals, 2000).

Defensins have a β-sheet structure, whereas histatins form amphipathic α-helices (Fig. 1 and 2). The mode of action of most linear α-helical peptides is comparable to that of the defensins. They have a net positive charge that attracts them to the anionic microbial surface. Their amphipathic structure favors insertion into microbial membranes, which results in permeabilization of the plasma membrane. These events eventually lead to loss of the intracellular contents and possibly lysis of the microorganism. However, extensive research over the past years on the mechanism of action of histatin 5 showed that the histatins have a mechanism of action that differs from that of most α-helical peptides. The mechanism of action of histatin 5 includes the binding of the peptide to a receptor, followed by the uptake of the peptide into the cell, cell cycle arrest, efflux of ATP out of the cell, targeting of the peptide to specific intracellular structures, and production of reactive oxygen radicals (ROS) (Kavanagh & Dowd, 2004). Unlike other antimicrobial proteins, histatins do not appear to lyse lipid membranes, as determined by release and dequenching of the fluorescent dye calcein. Analysis of the magnitude and time course of histatin-induced calcein release from *C. albicans* cells showed that loss of cell integrity was a secondary effect following cell death, rather than the result of primary disruption of the yeast cell membrane (Edgerton *et al.*, 1998).

Efforts to unravel the mechanism of action of histatin 5 in *C. albicans* has been going on for several years. Edgerton *et al.* (1998) reported the binding of ^{125}I-histatin 5 to a 67 kDa plasma membrane protein. This protein was later identified as Ssa1/2 (heat shock protein 70) (Li *et al.*, 2003). After binding to Ssa1/2, the peptide is internalized and targeted to the mitochondria (Helmerhorst *et al.*, 1999b). Once internalized, histatin 5 induces the efflux of ATP and potassium ions from the cell, G1 cell cycle arrest, and dysregulation of cell volume homeostasis, which is closely coupled with loss of intracellular ATP (Koshlukova *et al.*, 1999; Baev *et al.*, 2002). The histatin 5-induced ATP release occurs while the cells are still metabolically active and before any cell lysis occurs. The extracellular ATP is thought to interact with a purinergic receptor, which in turn induces cell death after activation (Koshlukova *et al.*, 2000).

The action of histatin 5 on *C. albicans* requires respiring cells and active mitochondria that perform oxidative phosphorylation (Helmerhorst *et al.*, 1999b). Petite cells of *C. albicans*, these cells are deficient in respiration due to mutations in mitochondrial DNA, are much more

resistant to histatin 5-induced cell death (Gyurko *et al.*, 2000). Furthermore, uncoupling or blocking of cellular respiration by carbonyl cyanide m-chlorophenylhydrazone (CCCP) or by azide protects against histatin 5-induced cell death because of the uncoupling of the respiratory chain phosphorylation (Koshlukova *et al.*, 1999). There is also a difference in susceptibility of the Crabtree-positive yeast *S. cerevisiae* for histatin 5, depending on whether it is grown on a fermentable or non-fermentable carbon source. For histatin 5 to exert its fungicidal action, active mitochondria must be present. When *S. cerevisiae* is grown aerobically on a non-fermentable carbon source (glycerol) the cells are respiring, resulting in a high oxidative phosphorylation rate and consequently a high susceptibility to histatin 5. Grown aerobically on glucose, a fermentable carbon source, *S. cerevisiae* cells will largely perform fermentation. Consequently, the mitochondria are not active and do not perform oxidative phosphorylation, which results in lower susceptibility to histatin 5. By contrast, *C. albicans*, a Crabtree-negative yeast, does perform oxidative phosphorylation on a fermentable carbon source under aerobic conditions, which makes it more sensitive to histatin 5-induced cell death. In *C. albicans*, oxidative phosphorylation is absolutely necessary for histatin 5-induced cell death. Although oxidative phosphorylation is important for histatin 5-induced cell death in *S. cerevisiae*, it is not a prerequisite. This difference points to an additional mechanism for histatin 5-induced cell death in *S. cerevisiae* that is not present in *C. albicans* (De Smet *et al.*, 2004).

Thus, the respiratory apparatus of the cell seems to be the target of histatin 5, resulting in inhibition of respiration, formation of radical oxygen species (ROS) and finally cell death due to the oxidation of biologically important molecules and the loss of cell integrity (Helmerhorst *et al.*, 2001). However, recent papers state that ROS play no essential role in the candidacidal activity of histatin 5 (Veerman *et al.*, 2004; Wunder *et al.*, 2004). The formation of ROS may be secondary to the effects of histatin 5 on cellular metabolism or ion homeostasis.

Further, potassium channels seem to play a role in histatin 5-induced cell death in *C. albicans*. Baev *et al.* (2003) first demonstrated the modulating role of the K^+ channel Tok1p in histatin 5-induced cell death. However, they concluded that Tok1p is not the primary site of histatin 5 action. Further work on the K^+ channel Trk1p showed that decreased expression of Trk1p resulted in significant resistance to histatin 5-induced cell death. The Trk1 protein seems to be the essential pathway for ATP loss, and is critical for the candidacidal activity of histatin 5 (Baev *et al.*, 2004). These recent papers emphasize the role of ATP release into the extracellular medium as critical for histatin 5-induced cell death, in contrast to the papers that stress the important role of mitochondria in the killing process. More research is necessary to elucidate the full molecular mechanism by which histatin 5 induces cell death in *C. albicans.*

Extensive clinical use of antibiotics has led to the growing emergence of strains of bacteria and fungi that are resistant to these commonly used drugs. Therefore, the development of a new class of antibiotics has become critical. Considerable effort is being invested in investigating

whether antimicrobial peptides or synthetic derivatives thereof can be used as therapeutic antibiotics. Further, the contraceptive potential of a few peptides has been explored, and some peptides have entered clinical trials for use in clinical applications other than the exploitation of their antimicrobial activity, such as the treatment of diabetic ulcers. From a range of studies it has become clear that antimicrobial peptides are implicated in several biological processes, and that they are an important component of the innate immune system. It is encouraging that a few of these peptides have the potential to be used as therapeutic drugs.

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Tables

Table 2. Amino acid sequence of four α-defensins, four β-defensins, the cathelicidin LL37, and the three most important histatins. Cysteine residues in the defensins are underlined.

Peptide	Sequence	Number of amino acids
α-defensins		
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	30
HNP-4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRVD	34
HD-5	ARATCYCRTGRCATRESLSGVCEISGRLYRLCCR	34
HD-6	RAFTCHCRRS-CYSTEYSYGTCTVMGN-HRFCCL	32
β-defensins		
hBD-1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK	36
hBD-2	DPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP	38
hBD-3	QKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	39
hBD-4	LDRICGYGTARCRKK-CRSQEYRIGRCPNTYA-CCLRKPWDESLLNRTK	47
Cathelicidin		
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37
Histatins		
Hst1	DSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN	38
Hst3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN	32
Hst5	DSHAKRHHGYKRKFHEKHHSHRGY	24

Table 3. Activity of antimicrobial peptides against Gram-positive and Gram-negative bacteria and some *Candida* species.

Peptide (family)	Organism	Strain	Gram-stain	MIC (µg/ml)
HNP-1 (α-defensin)[a]	*Listeria monocytogenes*	EGD	positive	39.7
	Staphylococcus epidermis		positive	5.2
	Staphylococcus aureus	502A	positive	2.2
	MRSA[f]	ATCC 33591	positive	21.2
	Bacillus subtilis		positive	6.4
	VREF[g]	CDC 21	positive	11.9
	Escherichia coli	ATCC 9637	negative	1.8
	Salmonella typhimurium	7953s	negative	8.4
	Pseudomonas aeruginosa	MR3007	negative	>250
	Burkholderia cepacia	ATCC 35416	negative	>250
	Stenotrophomonas maltophilia	411 A-15	negative	4.3
	Proteus mirabilis	ATCC 7002	negative	>250
	Proteus vulgaris	ATCC 13315	negative	>79.1
	Candida albicans	ATCC 820	yeast	>250
HBD-2 (β-defensin)[b]	*Peptostreptococcus micros*	ATCC 33270	positive	>250
	Actinomyces naeslundii	11A01	positive	8.2
	Actinomyces israelii	5A40	positive	9.1
	Streptococcus sanguis	NP506	positive	8.8
	Streptococcus mutans	ATCC 25175	positive	4.1
	Actinobacillus actinomycetemcomitans	ATCC 29523	negative	>250
	Fusobacterium nucleatum	ATCC 49256	negative	10.3
	Porphyromonas gingivalis	ATCC 33277	negative	34.6
	Escherichia coli	ATCC 9637	negative	3.7
	Candida tropicalis	II7	yeast	3.9
	Candida parapsilosis	ATCC 22019	yeast	17.8
	Candida krusei	ATCC 6258	yeast	12.2
	Candida glabrata	932474	yeast	22.7
	Candida albicans	ATCC 820	yeast	59.2
HBD-3 (β-defensin)[b]	*Peptostreptococcus micros*	ATCC 33270	positive	>250
	Actinomyces naeslundii	11A01	positive	7.2
	Actinomyces israelii	5A40	positive	9
	Streptococcus sanguis	NP506	positive	7.6
	Streptococcus mutans	ATCC 25175	positive	5
	Actinobacillus actinomycetemcomitans	ATCC 29523	negative	>250
	Fusobacterium nucleatum	ATCC 49256	negative	4.5
	Porphyromonas gingivalis	ATCC 33277	negative	5.7
	Escherichia coli	ATCC 9637	negative	5.1
	Candida tropicalis	II7	yeast	3.5
	Candida parapsilosis	ATCC 22019	yeast	12.4
	Candida krusei	ATCC 6258	yeast	2
	Candida glabrata	932474	yeast	33.8
	Candida albicans	ATCC 820	yeast	7.1
LL-37 (cathelicidin)[a]	*Listeria monocytogenes*	EGD	positive	1.5
	Staphylococcus epidermis		positive	7.6
	Staphylococcus aureus	502A	positive	3.6
	MRSA[f]	ATCC 33591	positive	3.4
	Bacillus subtilis		positive	2.7
	VREF[g]	CDC 21	positive	3.5
	Escherichia coli	ATCC 9637	negative	0.1
	Salmonella typhimurium	7953s	negative	0.4
	Pseudomonas aeruginosa	MR3007	negative	4.7
	Burkholderia cepacia	ATCC 35416	negative	>79.1
	Stenotrophomonas maltophilia	411 A-15	negative	1.9
	Proteus mirabilis	ATCC 7002	negative	5.7
	Proteus vulgaris	ATCC 13315	negative	2.5
	Candida albicans	ATCC 820	yeast	>250
P-113[e] (histatin)[c,d]	*Staphylococcus aureus*	102-0485	positive	12.5
	Pseudomonas aeruginosa	ATCC 19142	negative	3.1
	Burkholderia cepacia	cep0455	negative	>100
	Achromobacter xylosoxidans	8AU	negative	>100
	Stenotrophomonas maltophilia	61AT	negative	>100
	Candida albicans	ATCC 10231	yeast	3.1

	Candida glabrata	ATCC 98-2229	yeast	1.6
	Candida krusei	ATCC 14243	yeast	1.6
	Candida kefyr	ATCC 66028	yeast	6.3
	Candida parapsilosis	98-2318	yeast	3.1

[a] (Turner *et al.*, 1998)
[b] (Joly *et al.*, 2004)
[c] (Sajjan *et al.*, 2001)
[d] (Rothstein *et al.*, 2001)
[e] 12-amino acid fragment of histatin 5
[f] methicillin-resistant *Staphylococcus aureus*
[g] vancomycin-resistant *Enterococcus faecalis*

Figures

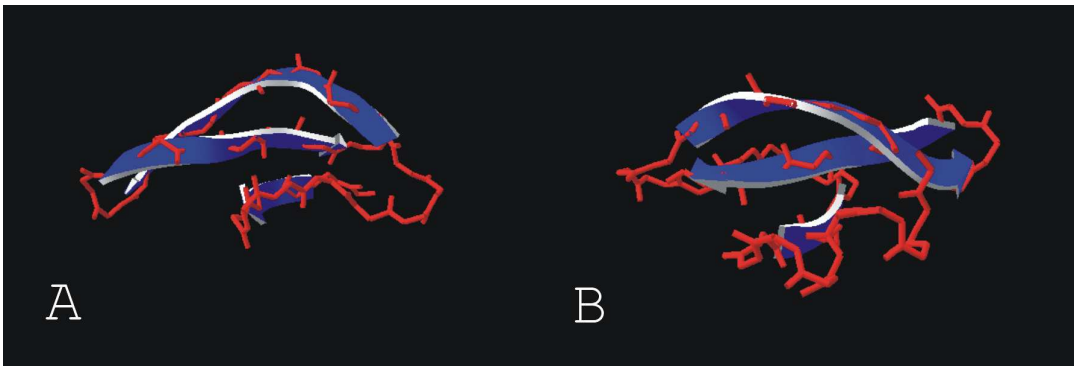

Figure 1. Three-dimensional models of the secondary structures of the α-defensin HNP-3 (A) and the β-defensin hBD-1 (B), generated using the programm Swiss-Pdb Viewer (Guex & Peitsch, 1997). The backbone of the peptide is in red, β-strands are indicated with blue ribbons.

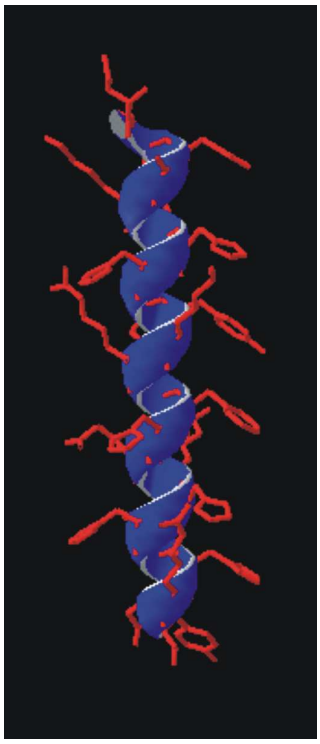

Figure 2. Theoretical 3D-model of the secondary structure of histatin 5, generated using the programm Swiss-Pdb Viewer (Guex & Peitsch, 1997). The backbone and sidechains of the peptide are in red, the α-helical structure is visualized in blue. The *N*-terminus is situated at the top and the *C*-terminus at the bottom.

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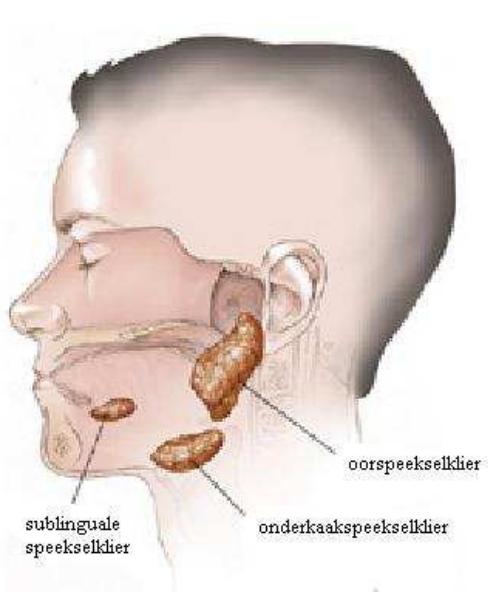
1.3 Histatine 5

1.3.1 De histatine familie

De verhoging van de metabole activiteit bij micro-organismen in de mond door elementen aanwezig in het speeksel werd reeds in de jaren vijftig geraporteerd (Hartles & Wasdell, 1954). Deze verhoging van de metabole activiteit heeft een invloed op de orale hygiëne, aangezien de microbiële glycolyse zorgt voor de aanwezigheid van een zuur milieu, dat verantwoordelijk is voor de initiatie en progressie van tandbederf. Men heeft vastgesteld dat verschillende proteïnen verantwoordelijk zijn voor deze verhoging van de metabole activiteit bij orale micro-organismen (Holbrook & Molan, 1973). Enkele van deze eiwitten bleken basische histidine-rijke peptiden te zijn. Hoewel ze mee verantwoordelijk zijn voor de vorming van een ongunstig, zuur milieu in de mond, toch bleken deze peptiden ook gunstige eigenschappen te hebben voor de orale hygiëne. Zij binden aan hydroxyapatiet, een belangrijke component van tandglazuur, en verhinderen de precipitatie van calciumfosfaatzouten in speeksel. Op deze manier helpen deze peptiden om een gunstig milieu in stand te houden in de mondholte om de tanden in goede gezondheid te houden. In de loop van de jaren hebben deze peptiden verschillende namen toegewezen gekregen: *histones*, *parotid basic* (Pb) en *post-parotid basic* (PPb) proteïnen, histidine-rijke basische factor, histidine-rijk zuur peptide, histidine-rijke proteïnen (HRP's), neutraal HRP en histatines (Holbrook & Molan, 1975). De algemeen aanvaarde benaming, histatines, werd voorgesteld in 1988 (Oppenheim *et al.*, 1988). Naast hun rol in het behoud van een gezonde mondhygiëne, werd het bovendien ook snel duidelijk dat de histatines een belangrijke functie hebben in het aangeboren immuunsysteem door hun bactericidale maar vooral ook hun fungicidale activiteiten (MacKay *et al.*, 1984b; Pollock *et al.*, 1984).

De histatines zijn kleine histidine-rijke cationische peptiden variërend in lengte van 7 tot 38 aminozuren. Ze worden gesecreteerd door de oor- en onderkaakspeekselklieren bij de mens en sommige hogere primaten (Fig. 3) en zijn in het speeksel aanwezig in een concentratie van 15-425 µM (Helmerhorst *et al.*, 1997).



Figuur 3: Ligging van de speekselklieren.

Door hun sterke antifungale activiteit is er veel onderzoek gebeurd naar hun eigenschappen, activiteit, structuur en secretie (Tsai & Bobek, 1998). Hoewel er tot voor kort 12 histatines beschreven werden, zijn er slechts twee genen gevonden die verantwoordelijk zijn voor de synthese van deze peptiden, *HIS1* en *HIS2*, gelegen op chromosoom 4q13 (Sabatini & Azen, 1989; vanderSpek *et al.*, 1989). *HIS1* en *HIS2* coderen respectievelijk voor histatine 1 en histatine 3. Histatine 1 is een peptide van 38 aminozuren, gefosforyleerd op positie Ser-2, met een moleculair gewicht van 4929 Da. Histatine 3 is 32 aminozuren lang en heeft een sequentie die sterk gelijkt op die van histatine 1, maar is niet gefosforyleerd en heeft een moleculair gewicht van 4063 Da. Beide peptiden bevatten 7 histidine-residuen, respectievelijk 18 en 29 % van de aanwezige aminozuren. Bij een vergelijking van de aminozuursequentie van beide peptiden blijkt dat de eerste 22 aminozuren gelijk zijn, met uitzondering van glutamaat op positie 4 en lysine op positie 11. Verschillende andere peptiden van deze familie werden geïsoleerd uit menselijk speeksel, allemaal hebben ze een sequentie gelijkend op de twee parentale peptiden. Hoewel verschillende classificaties zijn voorgesteld, gebruikt men nu deze voorgesteld door Troxler *et al.* (1990). Deze groep identificeerde in menselijk speeksel histatine 2, een peptide dat overeenkomt met de 27 C-terminale aminozuren van histatine 1, en negen andere peptiden, allemaal bestaande uit fragmenten van histatine 3. Deze peptiden worden histatine 4 tot en met 12 genoemd (Tabel 4). Behalve histatine 2, zijn alle kleinere histatines proteolytische producten van histatine 3.

Peptide	Sequentie	Lengte
Histatine 1	D-S_P-H-E-K-R-H-H-G-Y-R-R-K-F-H-E-K-H-H-S-H-R-E-F-P-F-Y-G-D-Y-G-S-N-Y-L-Y-D-N	38
Histatine 2	R-K-F-H-E-K-H-H-S-H-R-E-F-P-F-Y-G-D-Y-G-S-N-Y-L-Y-D-N	27
Histatine 3	D-S -H-A-K-R-H-H-G-Y-K-R-K-F-H-E-K-H-H-S-H-R-G-Y-R-S-N-Y-L-Y-D-N	32
Histatine 4	R-K-F-H-E-K-H-H-S-H-R-G-Y-R-S-N-Y-L-Y-D-N	21
Histatine 5	D-S -H-A-K-R-H-H-G-Y-K-R-K-F-H-E-K-H-H-S-H-R-G-Y	24
Histatine 6	D-S -H-A-K-R-H-H-G-Y-K-R-K-F-H-E-K-H-H-S-H-R-G-Y-R	25
Histatine 7	R-K-F-H-E-K-H-H-S-H-R-G-Y	13
Histatine 8	K-F-H-E-K-H-H-S-H-R-G-Y	12
Histatine 9	R-K-F-H-E-K-H-H-S-H-R-G-Y-R	14
Histatine 10	K-F-H-E-K-H-H-S-H-R-G-Y-R	13
Histatine 11	K-R-H-H-G-Y-K-R	8
Histatine 12	K-R-H-H-G-Y-K	7

Tabel 4: Aminozuursequenties van histatine 1 tot en met histatine 12 (S_P: fosfoserine).

Naast de 12 reeds gekende histatines konden recentelijk, door gebruik te maken van meer gevoelige scheidings- en detectiemethoden, een aanzienlijk aantal bijkomende peptiden geïdentificeerd worden in speeksel ontstaan door proteolyse van histatine 3 (Castagnola *et al.*, 2004). In verschillende studies konden echter, naast histatine 2, geen fragmenten afgeleid van histatine 1 gevonden worden (Xu *et al.*, 1992; Perinpanayagam *et al.*, 1995). Een belangrijk structureel verschil tussen deze twee peptiden is de fosforylatie van serine op positie twee bij histatine 1.

Het onderzoek naar histatines heeft zich hoofdzakelijk geconcentreerd rond histatine 5, een peptide bestaande uit de eerste 24 aminozuren van histatine 3. Histatine 5 is in de grootste concentratie aanwezig in het speeksel. Bovendien vertoont dit peptide de grootste specifieke antifungale activiteit tegen *Candida albicans* in vergelijking met de andere histatines (Xu *et al.*, 1991).

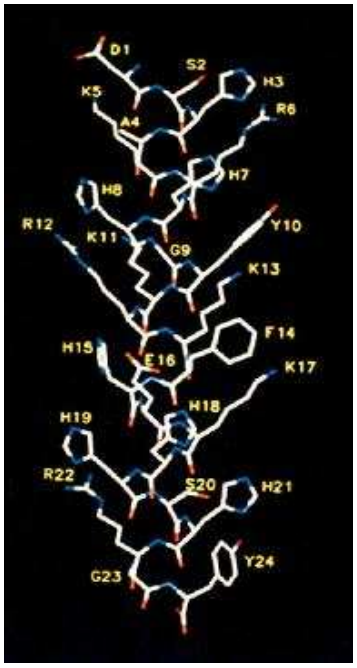
1.3.2 De structuur van histatine 5

De structuur van histatine 5 werd onderzocht aan de hand van circulair dichroïsme-spectra (CD-spectra), twee-dimensionele NMR-spectra en *molecular dynamics* (MD)-simulaties in hydrofiel en hydrofoob milieu (Raj *et al.*, 1990; Raj *et al.*, 1994; Raj *et al.*, 1998; Iovino *et al.*, 2001).

De CD-spectra werden opgenomen in waterige (water) en in niet-waterige solventen (trifluorethanol (TFE) en dimethylsulfoxide (DMSO)). De resultaten van deze studies toonden aan dat histatine 5 in een waterig solvent een *random coil* structuur aanneemt. Indien de CD-spectra werden opgenomen in een hydrofoob solvent, dan vertoonden de spectra

karakteristieken voor een peptide met een α-helicale structuur. De geordende helicale structuur van histatine 5 in een hydrofoob solvent wordt te niet gedaan door de intramoleculaire interacties tussen de CO- en NH-groepen van het peptide te breken. Dit gebeurt door de competitieve interactie van een zeer polair waterstof-bindend solvent (water). Histatine 5 blijkt dus structureel zeer flexibel (Raj *et al.*, 1990). Ook in dimyristoylfosfatidylcholine vesikels, een omgeving die de membraam nabootst, bleek histatine 5 een α-helicale conformatie aan te nemen (Fig. 4) (Raj *et al.*, 1994).

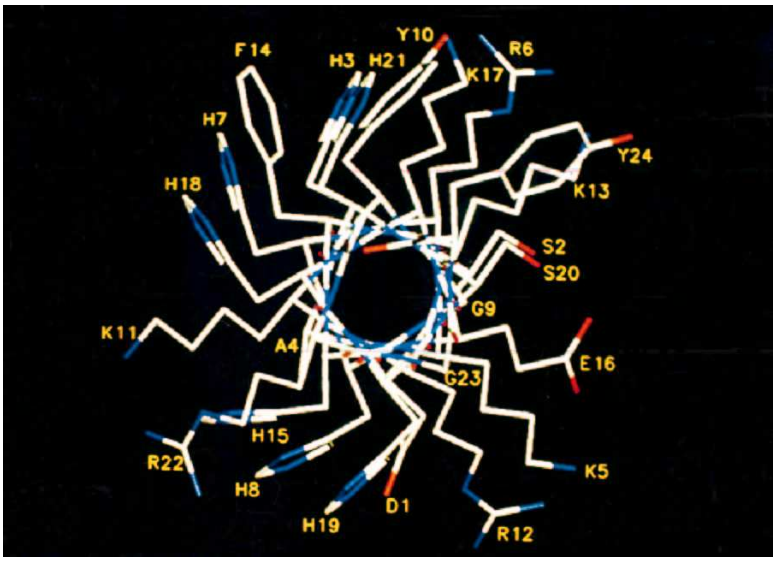
De resultaten bekomen met de NMR-studies (Raj *et al.*, 1994; Raj *et al.*, 1998) en de MD-simulaties (Iovino *et al.*, 2001) bleken de bevindingen van de CD-studies te bevestigen. Histatine 5 is zeer flexibel en ondergaat solvent-afhankelijke conformationele veranderingen. In een hydrofiel milieu neemt het een *random coil* structuur aan, in hydrofoob milieu neemt het een α-helicale structuur aan.



Figuur 4: Weergave van de α-helicale structuur van histatine 5 met bovenaan de N-terminus en onderaan de C-terminus (Raj *et al.*, 1998).

Verder blijkt dat, als de α-helicale structuur van histatine 5 langs de helix as wordt bekeken (Fig. 5), het peptide slechts zwak amfipatisch is. Een α-helix is amfipatisch als hydrofiele zijketens aan de ene kant van de helix zijn gelegen, terwijl hydrofobe zijketens aan de tegenovergestelde zijde zijn gelegen. Histatine 5 heeft slechts één hydrofoob residue (Phe 14) en partiële hydrofobiciteit is aanwezig in Tyr 10, Tyr 24, His 3, His 7, His 18 en His 21 aan de apolaire zijde van de α-helix (Raj *et al.*, 1998). Dit zwak amfipatische karakter van histatine 5

maakt het onwaarschijnlijk dat het peptide spontaan in microbiële membranen zal insereren en ionkanalen zal kunnen vormen. Op dit gebied verschilt histatine 5 sterk met andere antimicrobiële peptiden die voorkomen in de natuur, zoals bactenicines, defensines, magainines en tachyplesines. Deze cationische peptiden nemen een amfipatisch α-helicale of β-sheet structuur aan en kunnen spontaan in microbiële membranen insereren, om op deze manier ionkanalen te vormen en de cellulaire structuur te vernietigen (Lear *et al.*, 1988). Antibiotica die de structuur van de fungale cel vernietigen, zijn dikwijls ook toxisch voor menselijke cellen door hun structurele gelijkheid (eukaryote cellen). Histatine 5 blijkt echter niet toxisch te zijn voor mensen. Waarschijnlijk is dit te wijten aan het zwak amfipatische karakter van histatine 5, waardoor het niet spontaan in membranen plaats kan nemen.



Figuur 5: Weergave van de α-helicale structuur van histatine 5 langs de as van de helix, die het zwak amfipathische karakter toont, met de apolaire en de polaire zijde in respectievelijk de bovenste en de onderste helft (Raj *et al.*, 1998).

1.3.3 Werkingsmechanisme van histatine 5

1.3.3.1 Werkingsmechanisme van natuurlijke α-helicale lineaire antimicrobiële peptiden

Alhoewe sommige antimicrobiële peptiden een specifiek werkingsmechanisme bezitten, is het lyseren van de cel door permeabilisatie van de celmembraan het meest voorkomende mechanisme. Hierdoor verliest de cel intracellulaire componenten en sterft uiteindelijk. Voor de familie van de natuurlijke α-helicale lineaire antimicrobiële peptiden is aangetoond dat peptide-

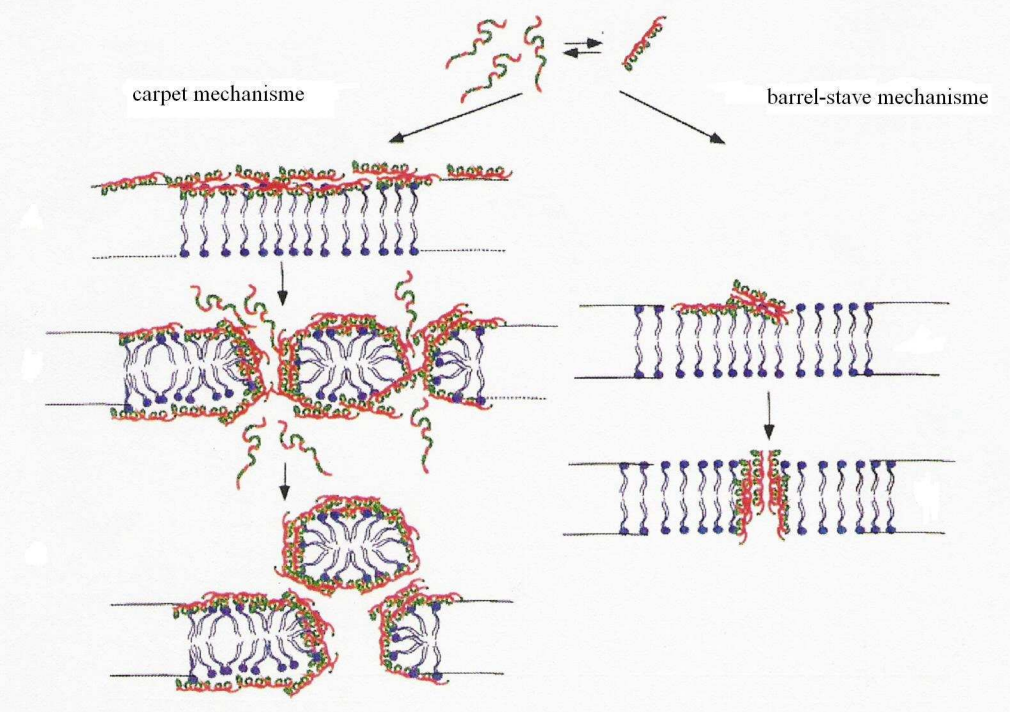
lipide interacties een belangrijke rol spelen en dat receptor-gemediëerde herkenning hier meestal niet van toepassing is. Dit werd aangetoond aan de hand van analogen van cecropine en magainine, die volledig opgebouwd zijn uit D-aminozuren. Deze peptiden bleken dezelfde antibacteriële activiteit als de natuurlijke peptiden te bezitten (Bessalle *et al.*, 1990; Wade *et al.*, 1990; Merrifield *et al.*, 1995). Bovendien bleek de amfipatische α-helicale structuur niet altijd noodzakelijk voor de peptiden om hun acitiviteit te kunnen uitoefenen (Shai & Oren, 1996). De antimicrobiële activiteit van de meeste peptiden van deze familie bestaat dan ook uit het lyseren van de cellen. Door de destabilisatie van de celmembraan verliest de cel zijn intracellulaire componenten wat uiteindelijk leidt tot celdood.

Membraanpermeabilisatie door amfipatische α-helicale peptiden kan verklaard worden aan de hand van (a) de vorming van transmembranaire poriën via het *barrel-stave* model (Ehrenstein & Lecar, 1977) of (b) membraandestructie via het *carpet-like* model (Pouny *et al.*, 1992; Gazit *et al.*, 1995) (Fig. 6).

- De vorming van een klassieke transmembranaire porie kan verklaard worden door het *barrel-stave* model. In het *barrel-stave* model vormen amfipatische α-helixen bundels waarbij hun hydrofobe oppervlakken interageren met de lipide kern van het membraan en hun hydrofiele oppervlakken naar binnen gericht zijn om zo een porie te vormen. Er zijn vier belangrijke stappen in dit mechanisme:
    1. De interactie tussen de α-helicale monomeren en het membraan. De binding aan het doelwitmembraan gebeurt voornamelijk op basis van hydrofobe interacties.
    2. Het samenvoegen van membraangebonden monomeren op basis van intramoleculaire herkennig tussen de monomeren. De herkenning tussen de antimicrobiële peptiden op het oppervlak van het membraan gebeurt reeds bij lage concentraties.
    3. De insertie in het membraan van tenminste twee verzamelde monomeren en de initiatie van een porie.
    4. Progressieve recrutering van additionele monomeren om de gevormde porie te vergroten.
- Een tweede mogelijk model is het *carpet-like* model. De amfipatische α-helicale peptiden binden initieel aan het oppervlak van het membraan en bedekken dit volledig of gedeeltelijk als een soort tapijt. De desintegratie van het membraan is enkel mogelijk als een bepaalde concentratie aan α-helicale peptiden is bereikt. De mogelijke stappen in dit model zijn:
    1. De preferentiële binding van de positief geladen peptide monomeren aan de negatief geladen fosfolipiden. De initiële interacties tussen de positief geladen α-helicale peptiden en de negatief geladen fosfolipiden zijn elektrostatisch van aard.

2. De amfipatische α-helicale monomeren leggen zich op het oppervlak van het membraan zodat de positieve ladingen van de basische aminozuren interageren met de negatief geladen fosfolipide hoofden.
3. Een rotatie van de molecule, die aanleiding geeft tot de reorientatie van de hydrofobe residuen van het α-helicale peptide naar de hydrofobe kern van de membraan.
4. Desintegratie van de membraan door het verstoren van de fosfolipidedubbellaag met de vorming van micellen als gevolg.

In tegenstelling tot het *barrel-stave* model, zal het peptide dus niet insereren in het hydrofobe deel van het membraan, maar eerder binden aan de negatief geladen hoofden van de fosfolipiden van het membraan en zo het membraan destabiliseren (Oren & Shai, 1998; Ruissen *et al.*, 2001).



Figuur 6: Voorstelling van het *carpet* (links) en *barrel-stave* (rechts) mechanisme voor membraanpermeabilisatie. De hydrofobe residuen van de peptiden zijn aangeduid in het groen, de hydrofiele zijn aangeduid in het rood (Oren *et al.*, 1998).

Beide modellen kunnen echter niet verklaren waarom sommige peptiden bacteriële membranen permeabiliseren in sub-lethale concentraties, of waarom sommige peptiden zeer bactericidaal zijn zonder dat zij een significante membraanpermeabilisatie vertonen. Deze observaties leidden tot het postuleren van het *aggregate-channel* model. Bij dit model clusteren kationische amfipatische peptiden samen ter hoogte van het membraanoppervlak op een min of meer

willekeurige manier. Kleine negatief geladen ionen en watermoleculen worden omgeven door de kationische amfipatische peptiden, waardoor de elektrostatische repulsie tussen de positief geladen zijketens van de peptiden vermindert. Deze clusters van peptiden destabiliseren dan lokaal het membraan en worden via een flip-flop mechanisme over het membraan gebracht. Op deze manier translokeren de peptiden over het membraan zonder een ernstige lekkage van de cel te veroorzaken. Hierbij veronderstelt men wel dat de meeste van deze peptiden intracellulaire targets hebben en dat de permeabilisatie van het microbieel membraan eerder een gevolg is, dan de primaire oorzaak van de anti-microbiële activiteit van de peptiden (Hancock, 1999; Ruissen *et al.*, 2001).

1.3.3.2 Werkingsmechanisme van histatine 5

Het werkingsmechanisme van histatine 5 is de laatste jaren sterk het onderwerp van onderzoek geweest en blijkt een proces te zijn dat uit meerdere stappen bestaat. Histatine 5 bindt op het celoppervlak eiwit Ssa1/2, en na internalisatie begeeft het zich naar de mitochondriën. Eens binnen in de cel induceert histatine 5 de efflux van ATP, kalium- en magnesiumionen in het extracellulair medium. De vrijstelling van ATP gebeurt terwijl de cellen nog metabool actief zijn en voor de cellen lyseren. Het vrijgestelde ATP zou op zijn beurt binden op een purinergische receptor, die na activatie celdood induceert. Verder induceert histatin 5 G1 celcyclus arrest en zorgt het voor een reductie in celvolume. Opdat histatine 5 zijn dodelijke functie op *C. albicans* zou kunnen uitoefenen, moeten de cellen respireren. Er moeten actieve mitochondriën, die oxidatieve fosforylatie uitvoeren, aanwezig zijn. Het cellulair doelwit van histatine 5 blijkt dus het respiratoir systeem van de cel, waar het zorgt voor inhibitie van respiratie en de vorming van zuurstof radicalen (ROS). Daarop volgt celdood wegens de oxidatie van biologisch belangrijke moleculen en het verlies van celintegriteit. Meer recente publicaties vermelden echter dat ROS geen rol spelen in het celdoodproces van histatine 5. De vorming van radicalen zou slechts een gevolg zijn van de effecten van histatine 5 op metabolische processen of ion homeostase. Bovendien blijken kaliumkanalen een rol te spelen in het histatine 5 geinduceerde celdood proces in *C. albicans*. De kaliumkanalen, Tok1 en Trk1, blijken een rol te spelen. In de volgende paragrafen zullen we dieper ingaan op elk van deze stappen in het histatine 5-geïnduceerde celdoodproces.

1.3.3.2.1 Effect van histatine 5 op de integriteit van membranen

Aangezien histatine een lineaire amfipatische α-helicale strutuur bezit, veronderstelde men dat histatine waarschijnlijk volgens het *carpet-like* model of het *barrel-stave* model getranslokeerd wordt over het membraan. Bovendien toonden electromicroscopische opnamen van *C. albicans* cellen, na een behandeling met histatine 5, zowel een beschadigd celmembraan als cytoplasmatische vacuoles, wat kan wijzen op verlies van intracellulaire componenten

(Santarpia *et al.*, 1990). Verder werd aangetoond dat *C. albicans* cellen kalium vrijstellen in het extracellulair milieu, na een histatine 5 behandeling (Pollock *et al.*, 1984). Later kwamen er echter experimentele data voorhanden die aantoonden dat membraanpermeabilisatie als primaire oorzaak van celdood onwaarschijnlijk is.

- De structuur van histatine werd gekarakteriseerd en toonde aan dat histatine een zwak amfipatisch karakter heeft. Opdat α-helicale peptiden zouden kunnen insereren in het membraan is een sterk amfipatisch karakter, dit wil zeggen een hoge graad aan hydrofobiciteit in additie aan de hydrofiele structuur, nodig om als peptide een kanaal te kunnen vormen in het membraan. Op basis van de structurele gegevens kan men dus besluiten dat het onwaarschijnlijk is dat histatine insereert in het membraan met vorming van een porie (Raj *et al.*, 1994; Helmerhorst *et al.*, 2001b).
- Aan de hand van confocale fluorescentiemicroscopie kon worden aangetoond met FITC-gemerkte peptiden dat de vorming van permanente transmembranaire multimere peptide poriën niet waarschijnlijk is. Tijdens de lokalisatie-experimenten werd er immers geen associatie van het histatine 5 met het cytoplasmatisch membraan vastgesteld. De peptiden werden geïnternaliseerd en interageerden met interne membranen (Ruissen *et al.*, 2001).
- Het effect van histatine 5 op de cel wordt bovendien gekenmerkt door de efflux van kalium en magnesium, waarbij de grootste hoeveelheid kalium- en magnesiumionen worden vrijgesteld 30 minuten na incubatie met histatine 5. Translokatie van histatine over het celmembraan gebeurt waarschijnlijk dus niet volgens een klassieke porie-vorming, omdat dit aanleiding zou geven tot een bijna directe vrijstelling van kalium en magnesium (Xu *et al.*, 1999).
- Het *C. albicans* membraan is ongeveer 70 Å (Voet & Voet, 1995), terwijl het histatine in helicale vorm slechts 36 Å lang is en het membraan dus zeker niet kan overspannen. Multimeren van histatine 5 of van het actief domein van histatine 5 zouden misschien wel in staat zijn om het membraan efficiënter te overspannen en bijgevolg meer candidacidale activiteit bezitten. Daarom werd de fungicidale werking van histatine multimeren nagegaan. De multimeren vertoonden geen verhoogde fungicidale activiteit, maar wel de neiging om α-helicale structuren te vormen in hydrofoob milieu. Verder toonden de CD-spectra aan dat histatine 5, zelfs bij concentraties 14 tot 18 maal boven zijn fysiologische concentratie, geen aggregaten vormde en in monomere toestand blijft bestaan. Op basis van deze experimentele data is het ook onwaarschijnlijk dat histatine in staat is om transmembranaire porieën te vormen (Situ *et al.*, 1999).
- Analyse van de tijdkinetiek en hoeveelheid van vrijstelling van de fluorescente kleurstof calceïne toonde aan dat het verlies van celintegriteit eerder een secundair effect is

volgend op celdood, dan het resultaat van een initiële permeabilisatie van het celmembraan (Edgerton *et al.,* 1998).

- Indien histatine voornamelijk een lytische activiteit zou bezitten dan zou directe vrijstelling van het celmembraan aan histatine een sterkere afdoding als gevolg moeten hebben. Wanneer sferoplasten echter behandeld werden met histatine kon geen sterkere afdoding vastgesteld worden, de sferoplasten bleken even gevoelig als de intacte cellen (Driscoll *et al.,* 1996). Sferoplasten aangemaakt door Edgerton *et al.* (1998) bleken zelfs 14 maal minder gevoelig te zijn vergeleken met intacte cellen. Hieruit blijkt dat ofwel een celwand component belangrijk is voor de fungicidale activiteit van histatine 5, ofwel worden de sferoplasten beschermd tegen histatine 5 door de verwijdering en/of verandering van membraancomponenten tijdens de vorming van de sferoplast (Edgerton *et al.,* 1998).

Deze waarnemingen tonen aan dat het onwaarschijnlijk is dat het histatine 5 poriën vormt in het plasmamembraan van de cel en op deze manier celdood veroorzaakt. Het peptide zal dus niet kunnen internaliseren via het *barrel-stave* of *carpet-like* mechanisme, het *aggregate-channel* mechanisme blijft echter wel een mogelijke manier voor internalisatie van histatine 5.

1.3.3.2.2 Receptor-gemedieerde opname

Aan de hand van een *overlay-assay* met radioactief gemerkt histatine 5, kon men een 67 kDa histatine 5-bindend eiwit detecteren. Bovendien, kon met *cross-linking* experimenten het bestaan van een 73-kDa ^{125}I-histatine 5-bevattend complex aangetoond worden. Indien hetzelfde experiment werd uitgevoerd met *S. cerevisiae* werd een dubbele band ontdekt met relatieve massa's van ongeveer 70 en 87 kDa. De sneller migrerende fractie is van dezelfde grootte als het histatine 5-bindende eiwit in *C. albicans*. De 87 kDa fractie kan dit histatine 5-bindende eiwit zijn, met een of twee molecule ^{125}I-histatine 5 gebonden.

Op basis van de gegevens bekomen door de *overlay-assay* werd gesuggereerd dat er een eiwit aanwezig is in *C. albicans* dat specifiek histatine 5 bindt. Dit eiwit zou in het membraan van *C. albicans* als receptor voor histatine kunnen fungeren (Edgerton *et al.,* 1998).

Om dit histatine-bindend eiwit te identificeren werd gebruik gemaakt van kolomchromatografie van volledige cellysaten van *C. albicans* en *matrix-assisted laser desorption ionization mass spectrometry* (MALDI-MS). Het eiwit werd geïdentificeerd als het het *heat shock* eiwit Ssa1/2. Aan de hand van ondermeer *yeast two-hybrid* analyse en colokalisatie experimenten werd de interactie tussen histatine 5 en Ssa1/2 bevestigd. *S. cerevisiae ssa1/ssa2* mutanten bleken bovendien een verminderde binding van histatine 5 en een verminderde gevoeligheid voor histatine 5 te vertonen (Li *et al.,* 2003). Ssa eiwitten maken deel uit van de HSP70 familie die hun functie hebben in bescherming tegen hitteschok, als hulp bij eiwit opvouwing en bij translocatie langs membranen. Ssa1 en Ssa2 zijn belangrijke immunogenen van *C. albicans* die

gelegen zijn in de celwand en een cel-gemedieerde immuniteitsreactie uitlokken in muizen en mensen die gekoloniseerd zijn met *C. albicans*. Deze uitgesproken immunogene eigenschappen zijn mogelijk door de lokalisatie aan de buitenkant van de cel en bevestigen hun mogelijkheid om als receptoren op te treden (Li *et al.*, 2003).

1.3.3.2.3 Intracellulaire expressie van histatine 5

Om het belang van extracellulaire binding en translocatie van histatine na te gaan, werden stammen van *C. albicans* gecreëerd die genen van histatine 3 of 5 geïntegreerd hebben in hun genoom onder controle van een induceerbare promotor. Inductie van intracellulaire expressie van zowel histatine 3 als histatine 5 in deze stammen zorgde voor een verlies van celviabiliteit en voor efflux van ATP onafhankelijk van extracellulaire binding en translocatie. Net zoals voor celdood geïnduceerd door extern toegediend histatine, vertonen cellen die histatine 5 expresseren een grotere graad van cytotoxiciteit dan cellen die histatine 3 expresseren. Aangezien intracellulaire componenten op zich histatine-geïnduceerde celdood in gang kunnen zetten, zijn extracellulaire binding en translocatie over het cytoplasmatisch membraan slechts transportgebeurtenissen (Baev *et al.*, 2001). Dit heeft als gevolg dat binding van histatine op een eventuele receptor (bv. Ssa1/2) niet noodzakelijk is om celdood te induceren. De peptiden zouden de cel ook via het *aggregate-channel* model kunnen binnendringen en op deze manier de intracellulaire componenten bereiken die het celdoodproces in gang steken.

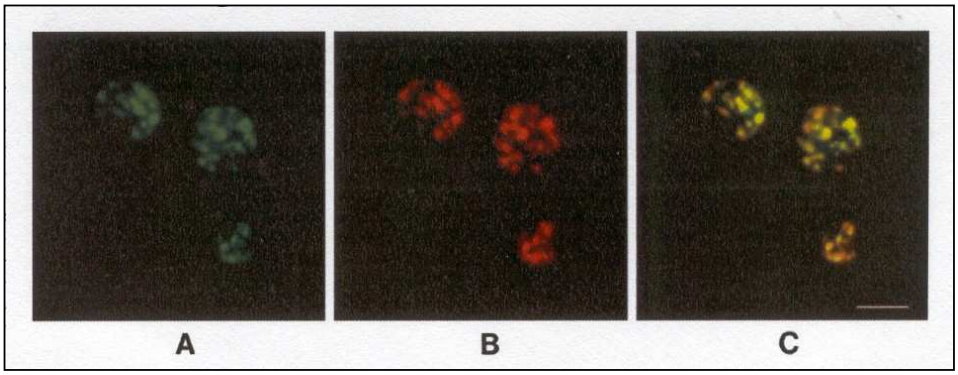
1.3.3.2.4 Rol van de mitochondriën in het histatine 5-geïnduceerde celdoodproces in C. albicans

Een interessante vaststelling die gedaan werd is dat histatine 5 niet in staat bleek om *C. albicans* cellen te doden bij een temperatuur van 4°C, wat erop wijst dat de afdodingsactiviteit van histatine afhankelijk kan zijn van de metabole activiteit van de cel. Om dit na te gaan werden afdodingstesten gedaan met *C. albicans* onder condities dat de cel niet in staat is om mitochondriaal ATP aan te maken. In gist kan oxidatieve fosforylatie geblokkeerd worden door het verhinderen van mitochondriale ATP synthese met specifieke inhibitoren of door het induceren van mutaties in het respiratoir systeem. Natriumazide inhibeert cytochroom oxidase waardoor de cellen beperkt zijn tot niet-oxidatieve pathways voor ATP synthese. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), een ontkoppelaar van de membraan protongradient, stopt ATP synthese zonder hierbij de zuurstof opname van de cel te blokkeren. Verder werden mutante stammen van *C. albicans* gecreëerd die deficient zijn in mitochondriale respiratie, zogenaamde petite cellen (Gyurko *et al.*, 2000). Deze mutanten kunnen enkel groeien op fermenteerbare koolstofbronnen, zoals glucose of fructose, en niet op ethanol of glycerol (Hatab & Whittaker, 1992). Cytochroom bepalingen toonden aan dat petite mutanten ofwel cytochroom *b* of cytochroom aa_3 of beide missen, maar nooit cytochroom *c* (Sherman & Slonimski, 1964).

De moleculaire basis van het petite fenotype is een mutatie in of deletie van mitochondriale genen (Faye *et al.*, 1973).

De petite mutanten van *C. albicans* bleken een sterk verminderde gevoeligheid te vertonen voor histatine 5 vergeleken met de wild type cellen. Ook cellen die behandeld werden met natriumazide of CCCP, of cellen die in anaerobe omstandigheden gegroeid werden, bleken een verhoogde resistentie te vertonen voor histatine 5-geïnduceerde celdood. Deze reductie in afdodingsactiviteit bleek samen te gaan met een verminderde opname van histatine 5 door de cellen. De opname is dus afhankelijk van de metabole staat van de cel en is direct gerelateerd met de fungicidale activiteit van histatine 5. Niet-respirerende *Candida* cellen internaliseren het peptide veel minder en zijn beschermd tegen histatine 5-geïnduceerde celdood (Helmerhorst *et al.*, 1999b; Gyurko *et al.*, 2000). Men kan besluiten dat energie-afhankelijke opname van histatine 5 noodzakelijk is voor het doden van *C. albicans* cellen. De opname van histatine 5 bleek echter wel samen te gaan met de internalisatie van propidium jodide (Helmerhorst *et al.*, 1999b). Indien histatine 5 opname receptor gemedieerd is (bv. via Ssa1/2), dan kan het zijn dat een deel van het peptide toch internaliseert via interactie met de lipidebilaag. Deze internalisatie zou dan kunnen gebeuren volgens het *aggregate-channel* model.

Een directe betrokkenheid van de mitochondriën in het afdodingsproces werd aangetoond door colokalisatie van fluorescent gemerkt histatine 5 (FITC-histatine 5) met een mitochondriale specifieke probe (Mitotracker Orange). Histatine 5 bleek een granulaire intracellulaire distributie te vertonen, wat overeenkomt met de distributie van de mitochondriën (Fig. 7) (Helmerhorst *et al.*, 1999b; Ruissen *et al.*, 2001).



Figuur 7: Colokalisatie van FITC-gemerkt histatine 5 met een mitochondriale merker (Mitotracker Orange) in *C. albicans* cellen. A: lokalisatie van FITC-histatine 5. B: lokalisatie van mitochondriale merker. C: dubbele labelling. Staaf = 5 µm (Helmerhorst *et al.*, 1999b).

Door meting van de vrijstelling van de potentiaal-afhankelijke merker rhodamine 123 werd aangetoond dat blootstelling van de mitochondriën aan histatine 5 resulteert in een verlies van mitochondriale transmembraanpotentiaal (Helmerhorst *et al.*, 1999b). Aangezien de

mitochondriale *targetting* samen gaat met verlies van membraanpotentiaal, wijst dit op een schadelijk effect voor de mitochondriale functie op een bepaald ogenblik in het celdoodproces door middel van een direct of indirect mechanisme.

Uit de bovenstaande bevindingen kunnen volgende stappen in het histatin 5-geïnduceerde celdoodproces afgeleid worden: interactie van histatine 5 met het cytoplasmatisch membraan van de cel (rechtstreeks of binding op een receptor), translocatie over het cytoplasmatisch membraan, vrijstelling in het cytoplasma, gevold door *targetting* naar mitochondriën en permeabilisatie van het mitochondriaal membraan met verlies van mitochondriale membraanpotentiaal

1.3.3.2.5 Zuurstofradikalen

De sterk verminderde gevoeligheid van *Candida* cellen voor histatine 5 die verkregen wordt door de cellen anaeroob of in de aanwezigheid van inhibitoren van de respiratieketen op te groeien, toont aan dat cellulaire respiratie noodzakelijk is opdat histatine 5 zijn werking kan uitvoeren. Alhoewel verschillende fenomenen beschreven werden die gebeuren na blootstelling van gistcellen aan histatine 5, was het nog steeds onduidelijk wat de belangrijke gebeurtenissen zijn en wat nu de eigenlijke oorzaak is van de celdood. Daarom werden in meer detail de interactie van histatine 5 met de mitochondriën en de biologische gevolgen van deze interactie bestudeerd (Helmerhorst *et al.*, 2001a). Twee belangrijke bevindingen werden geformuleerd. Ten eerste bleek dat histatine 5 optreedt als respiratorische inhibitor, en was er een zeer sterke correlatie tussen inhibitie van respiratie en afdoding van de cellen. Het was echter nog niet duidelijk hoe blokkering van de respiratieketen kan leiden tot celdood, aangezien *Candida* volledig fermentatief kan groeien. Zo zijn hoge concentraties van respiratie inhibitoren (azide, cyanide, antimycine) niet fungicidaal (Shepherd *et al.*, 1978). Ten tweede toonden fluorimetrische metingen (oxidatie door ROS van dihydroethidium tot zijn fluorescent derivaat) aan dat, na blootstelling aan histatine 5, er zuurstofradicalen (*reactive oxygen species*, ROS) gevormd worden in *C. albicans* cellen en geïsoleerde mitochondriën. De hoeveelheid ROS was bovendien sterk gecorreleerd met celdood. In de aanwezigheid van een zuurstof *scavenger* (L-cysteine) werd celdood en ROS-vorming vermeden. Bovendien kon histatine 5-geïnduceerde ROS-vorming in geïsoleerde mitochondriën vermeden worden door 2,2,6,6-tetramethylpiperidine-*N*-oxyl, een molecule dat de werking van superoxide dismutase nabootst en membranen kan passeren. Er is dus sterk bewijs geleverd dat histatine 5-geïnduceerde celdood gepaard gaat met ROS-vorming en dat dit een essentiële stap in het proces is (Helmerhorst *et al.*, 2001a). De specifieke plaats waar histatine 5 zijn werking uitvoert moet nog bepaald worden. Er werd aangetoond dat mitochondriale ROS gevormd worden zowel aan het NADH/ubiquinon reductase (complex I) als aan het ubiquinol/cytochroom *c* reductase (complex III) (Wolvetang *et al.*, 1994; Li *et al.*, 1999).

Wunder *et al*. bevestigden dat de totale cellulaire hoeveelheden ROS in *C. albicans* verhogen na de exogene toediening van histatine 5, zoals door Helmerhorst *et al*. werd aangetoond. Bovendien konden zij ook een verhoging van ROS vaststellen na intracellulaire expressie van histatine 5. De detectie van ROS gebeurde door gebruik te maken van de oxidatieve-gevoelige probe H_2-dichloro-dihydrofluoresceine diacetaat. Ondanks deze verhoging van ROS kon er geen proteïnecarbonylatie gedetecteerd worden (Wunder *et al.*, 2004). De respiratieketen genereert ROS (superoxide radicalen) die membraanlipiden en cellulaire proteïnen oxideren en op deze manier proteïnecarbonylatie veroorzaken. In *S. cerevisiae* is de graad van proteïnecarbonylatie sterk verbonden met de graad van ROS vorming (Aguilaniu *et al.*, 2001).

Alhoewel histatine 5-geïnduceerde celdood sommige kenmerken van apoptose vertoont, zoals G_1-celcyclus arrest (Baev *et al.*, 2002) en ROS vorming, konden geen enkele andere kenmerken van apoptose, zoals proteïnecarbonylatie, DNA fragmentatie en cytochroom *c* vrijstelling uit de mitochondriën, aangetoond worden. Bovendien bleek een voorbehandeling van *C. albicans* cellen met cycloheximide niet te resulteren in bescherming tegen histatine 5-geïnduceerde celdood (Edgerton *et al.*, 1998), wat aantoont dat er geen expressie is van pro-apoptotische proteïnen die nodig zijn om de celdoodcascade te initialiseren. Verder werd aangetoond dat superoxide dismutase (SOD) mutanten van *S. cerevisiae* of *C. albicans* niet gevoeliger zijn voor histatine 5 als wild-type cellen. De data gepubliceerd door Wunder *et al.* suggereren dat de vorming van ROS in histatine 5 behandelde cellen een secundair effect is, naast het voornaamste toxische effect van histatine 5, en dat histatine 5-geïnduceerde celdood niet via een apoptotisch proces gebeurt (Wunder *et al*., 2004).

Ook door Veerman *et al.* werd dihydroethidium gebruikt als een indicator voor ROS om in het histatine 5-geïnduceerde celdoodproces ROS vorming aan te tonen. Alhoewel zij ook een sterke stijging in fluorescentie konden waarnemen, kon deze stijging niet verhinderd worden door 2,2,6,6-tetramethylpiperidine-*N*-oxyl. Deze ROS *scavenger* kon ook celdood niet verhinderen. Zij concludeerden dat er geen ROS gevormd worden en dat ROS geen rol spelen in het histatine 5-geïnduceerde celdoodproces in *C. albicans*. De stijging in fluorescentie weten ze aan de vrijstelling van ongebonden, vooraf gevormd ethidium vanuit de mitochondriale matrix in het cytoplasma (Veerman *et al.*, 2004).

De vorming en rol van ROS in histatine 5-geïnduceerde celdood in *C. albicans* is dus nog een onderwerp van discussie. Verder onderzoek zal moeten uitwijzen of en in welke mate ROS van belang zijn in dit celdoodproces.

1.3.3.2.6 Vrijstelling van intracellulaire componenten in het extracellulair milieu

Histatine 5 induceert in *C. albicans* depletie van intracellulaire ATP-niveaus en als gevolg daarvan het stijgen van de extracellulaire concentratie aan ATP. We hebben reeds eerder vermeld dat inhibitoren en ontkoppelaars van cellulaire respiratie, en de incubatie onder anaerobe toestand de cellen beschermen tegen de fungicidale activiteit van histatine 5. Bij het bepalen van intracellulaire ATP-niveaus stelde men vast dat cellen, waarbij de mitochondriale activiteit verhinderd werd, geen depletie van de intracellulaire ATP-niveaus vertoonden. Men stelde ook vast dat de extracellulaire ATP-niveaus niet toenamen bij deze metabool inactieve cellen. De ATP-efflux werd dus verhinderd bij cellen waarvan de mitochondriale activiteit was verhinderd (Koshlukova *et al.*, 1999).

Op basis van experimentele data kon het celdodende effect van histatine 5 worden gecorreleerd met de efflux van ATP, en de depletie van intracellulaire ATP-niveaus. De depletie van intracellulair ATP is afhankelijk van de concentratie van en de incubatietijd met histatine 5. De maximale ATP-efflux bij incubatie van de cellen met histatine bij een fysiologische concentratie van 31 µM werd vastgesteld na 30 minuten, dit moment kwam overeen met de maximale reductie van intracellulaire ATP-niveaus en ook met de maximale reductie in celviabiliteit (Koshlukova *et al.*, 1999).

De vrijstelling van ATP uit actieve *C. albicans* cellen gebeurt volgens een niet-lytisch mechanisme. Dit werd aangetoond door verschillende vaststellingen:

- We vermeldden reeds dat het histatine een verstoring van de membraanpotentiaal tot gevolg heeft. Er werd nagegaan of er een verband is tussen ATP-depletie, de celdood van *C. albicans* en de depolarisatie van de celmembranen. Men stelde vast dat *C. albicans* cellen tot 90 minuten na de vrijstelling van ATP (ATP werd vrijgesteld vanaf het begin van incubatie van de cellen met histatine) respiratorisch actief bleven en gepolariseerde membranen hadden. Men stelde vast dat de fluorescentie-intensiteit van een fluorescente kleurstof ($DiOC_5$) in de cel, niet verlaagde tot 90 minuten na behandeling van de cellen met histatine, wat dus wijst op het behoud van gepolariseerde celmembranen.
- Verder bleek dat er geen afname was van de extracellulaire ATP-gehaltes gedurende de eerste 80 minuten. Dit suggereert dat er geen intracellulaire ATPasen vrijkomen die het ATP kunnen afbreken. Intracellulaire ATPasen worden pas vrijgesteld na desintegratie van het celmembraan.
- De met histatine behandelde cellen consumeren zuurstof aan een snelheid die gelijkaardig is aan deze van niet behandelde cellen.

Deze argumenten samen tonen aan dat de ATP-efflux gebeurt uit actieve, intacte cellen en niet gaat volgens een lytisch mechanisme (Koshlukova *et al.*, 1999).

Hoe gebeurt nu de vrijstelling van ATP uit de cel? Er zijn enkele pathways voor de specifieke vrijstelling van ATP beschreven: 1) vergemakkelijkt transport van ATP via een transporter met een concentratie gradient mee, 2) transport door een ATP-specifiek of anion transportkanaal en 3) vrijstelling van ATP door exocytose (Schwiebert *et al.*, 1998). In dit geval zou de vrijstelling van ATP kunnen gebeuren via een pathway die gebruik maakt van een transportkanaal. Mogelijke kandidaten zijn ATP-binding cassette (ABC) eiwitten. Verschillende ABC-transporters werden beschreven die betrokken zouden zijn bij het transport van ATP, zoals P-glycoproteïne (Roman *et al.*, 1997) en *cystic fibrosis conductance regulator* (Schwiebert *et al.*, 1995).

In hogere eukaryote cellen spelen vrijgestelde purinenuleotiden en -nucleosiden een verdere rol door interactie met purinergische oppervlaktereceptoren, die na activatie een reeks van biologische effecten veroorzaken. Op basis hiervan veronderstelde men dat het vrijgestelde ATP membranaire receptoren activeert die gelijkaardig zijn aan de P2 purinergische receptoren en dat deze receptoren na activatie een invloed hebben op viabiliteit van de cel (Surprenant *et al.*, 1996). Om dit te testen werden *C. albicans* cellen geïncubeerd met purinergische agonisten en antagonisten. Na incubatie met de agonisten, ATP-analogen, werden *C. albicans* cellen gedood, terwijl incubatie met de antagonisten een histatine 5-geïnduceerde celdood verhinderde. Deze resultaten tonen aan dat er oppervlaktereceptoren zijn op het *C. albicans* membraan die gelijkaardig zijn aan de purinergische oppervlaktereceptoren en dat het vrijgestelde ATP na incubatie van de cellen met histatine 5 als ligand voor deze oppervlaktereceptoren celdood van *C. albicans* induceert. De vrijstelling van het ATP gaat het signaal van cytotoxiciteit vooraf. Het eigenlijke signaal voor cytotoxiciteit zou gemedieerd worden door de extracellulaire ATP-niveaus (Koshlukova *et al.*, 2000).

Om de *Candida* $P2X_7$-gelijkaardige receptor te vinden werd de membranaire fractie van het *C. albicans* membraan onderzocht. Een 60 kDa membranair eiwit werd gedetecteerd in *C. albicans* door middel van anti-$P2X_7$ serum van konijn. Dit 60 kDa proteïne is een kandidaat als $P2X_7$-gelijkaardige receptor (Koshlukova *et al.*, 2000).

Naast de ATP depletie is er ook vrijstelling van kalium- en magnesiumionen na blootstelling van *C. albicans* cellen aan histatine. De extracellulaire concentratie aan magnesium en kalium bleek sterk te stijgen na incubatie van de cellen met histatine 3. Vijf minuten na het begin van incubatie start de efflux van kalium uit de *Candida* cel, efflux van magnesium kan na 15 minuten waargenomen worden. Histatine 3-gemedieerd verlies van kalium en magnesium bereikt een maximum na 30 minuten, wat overeenkomt met het tijdsverloop van celdoding (Xu *et al.*, 1999).

1.3.3.2.7 Verstoring van volume regulatie en blokkeren van celcyclus

In hogere eukaryote cellen veroorzaakt hypotone stress ATP vrijstelling, wat een essentieel controlemechanisme is voor celvolume regulatie (Wang *et al.*, 1996). De opvallende vrijstelling van ATP na histatine 5-behandeling van *C. albicans* cellen deed de vraag rijzen of deze ATP vrijstelling gepaard gaat met veranderingen in celvolume. Door Baev *et al.* (2002) werd aangetoond dat histatine 5-geïnduceerde celdood gepaard gaat met een vermindering in cellulair volume. Bovendien konden ze aantonen dat inhibitoren van iontransport of volume-regelende Cl^--kanalen een belangrijke resistentie verlenen tegen histatine 5-geïnduceerde celdood en tegen histatine 5-geïnduceerde celvolume reductie. Aangezien ATP vrijstelling en een snelle vermindering in volume van de cellen volledig kan geblokkeerd worden door een voorbehandeling van de cellen met een inhibitor van iontransport (DIDS, *diisothiocyanatostilbene-2,2'-disulfonic acid*), wijst dit erop dat aniontransporters belangrijke componenten zijn in de verstoring van de celvolume regulatie die gepaard gaat met histatine 5-geïnduceerde celdood. Gist membraantransporters bezitten een goed gedefinieerde rol in volume regulatie. Cellen verminderen hun volume door de efflux van de Cl^- en K^+ ionen, wat gepaard gaat met de efflux van water uit de cel. Een snelle efflux van kalium en magnesium uit de *C. albicans* cellen gebeurt na incubatie met histatine 5 (Xu *et al.*, 1999), wat erop wijst dat ion efflux een initiërende gebeurtenis is. Het snelle volumeverlies kan erop wijzen dat histatine 5 transporters van anionen activeert parallel met de efflux van K^+ uit de *C. albicans* cellen. Gisten initiëren geen nieuwe ronde van celdeling indien er niet aan bepaalde vereisten voldaan is. Een van deze vereisten is een bepaalde celgrootte/celvolume verhouding. Gistcellen gaan slechts naar Start in de celcyclus (ook het Restrictiepunt genoemd) indien een bepaalde kritische grootte bereikt is (Futcher, 1996; Planas-Silva & Weinberg, 1997). De histatine 5-geïnduceerde verstoring van iontransport en celvolume homeostase verhindert volume toename van de gistcellen, kritiek in de celcyclus van gisten, en leidt dus tot G_1 arrest (Baev *et al.*, 2002).

1.3.3.2.8 De rol van kaliumkanalen in het histatine 5-geïnduceerde celdood proces

De actie van histatine 5 op *C. albicans* cellen gelijkt in meerdere opzichten op de actie van K1 killer toxine op *S. cerevisiae*. Beiden werken via een receptor-gemedieerd proces dat uit meerdere stappen bestaat. K1 toxine bind initieel op Kre9, een receptor in de celwand van *S. cerevisiae* (Breinig *et al.*, 2002), waarna er vrijstelling is van kalium en ATP in het extracellulair milieu. Er is aangetoond dat de activiteit van K1 killer toxine gedeeltelijk gericht is tegen de Tok1 in *Saccharomyces*, een kaliumkanaal (Ahmed *et al.*, 1999). Verder werd door de bevindingen van Baev *et al.* (2002) in het onderzoek naar het celdoodmechanisme van histatine 5 de aandacht gevestigd op de rol van de kaliumkanalen in het celdoodproces. De vraag of het *Candida* homoloog van het *Saccharomyces* Tok1 een doelwit zou zijn van histatine

5 actie werd onderzocht door het maken van *Candida* stammen waarbij *TOK1* gedeleteerd is. Deletie van *TOK1* verhoogde de overleving van cellen na een histatine 5 behandeling en zorgde voor een verminderde efflux van ATP. Toch bleef er een groot deel van de cellen gevoelig voor histatine 5 en was er nog een substantieel verlies van ATP. Dit wijst erop dat Tok1 niet de voornaamste plaats is waar histatine 5 op inwerkt, maar dat dit kaliumkanaal toch een modulerende rol speelt (Baev *et al.*, 2003). De mogelijke invloed van een ander kaliumkanaal, Trk1, werd onderzocht door overexpressie van het Trk1 eiwit door de introductie van een extra *TRK1* gen en door de constructie van een *single-allele-deletion strain* (Baev *et al.*, 2004). Het bleek onmogelijk om beide *TRK1* allelen te deleteren, wat erop wijst dat dit een essentieel gen is. De *single-allele-deletion strain* bleek sterk gereduceerde hoeveelheden van functioneel Trk1 te expresseren, en bleek sterk verminderde toxische effecten van histatine 5 te ondervinden (zowel celdood als ATP vrijstelling). Wat op een sterke invloed wijst van Trk1 op het celdood proces geïnduceerd door histatine 5. De overexpresserende stam bleek echter tegenstrijdige resultaten te leveren. Ondanks dat er een 50% verhoging was van de Trk1 activiteit, bleek er een 50% verlaging in histatine 5 gevoeligheid op te treden. Door de auteurs werden hiervoor twee verklaringen gegeven. Een eerste mogelijkheid is dat de overmaat aan Trk1 opgeslagen wordt in endomembranen om de dreiging van overpermeabilisatie van het plasmamembraan tegen te gaan. Indien er een directe interactie is tussen histatine 5 en Trk1 zou een verhoogde endomembranaire Trk1 concentratie een aanzienlijke hoeveelheid histatine 5 kunnen binden en op deze manier de vrije concentratie doen dalen. Er zou dan minder vrij histatine 5 aanwezig zijn dat op het functionele, plasmamembranair Trk1 kan binden en zijn fungicidale activiteit kan uitoefenen. Een tweede verklaring die gegeven werd is dat het plasmamembranair Trk1 in verschillende oligomerische vormen voorkomt, waarvan er slechts enkele een interactie met histatine 5 kunnen aangaan. Er zouden drie verschillende oligomeren kunnen voorkomen van de transporter: 1) vrij Trk1, een niet-functionele vorm die geen histatine 5 bindt, 2) Trk1 gebonden aan een activator, een functionele vorm die histatine bindt en 3) Trk1 gebonden aan twee activatoren, een functionele vorm die geen histatine 5 bindt. Het bestaan van verschillende oligomeren van Trk1 is door verschillende bronnen gesuggereerd (Schlosser *et al.*, 1993; Durell *et al.*, 1999; Harms *et al.*, 2001; Zeng *et al.*, 2004). De deletie van één Trk1 allel zorgt voor een belangrijke verhoging van de resistentie voor histatine 5 geïnduceerde celdood. Daarentegen induceert overexpressie van dit eiwit, wat resulteert in 50% verhoging van Trk1 functionaliteit, eveneens een verhoogde resistentie. Verder onderzoek naar de rol van de kaliumkanalen in het celdoodproces geïnduceerd door histatine 5 kan een antwoord bieden op de nog openstaande vragen.

1.3.4 Synthetische derivaten van histatine 5

In de loop van de jaren werden voor verschillende onderzoeksdoeleinden een groot aantal varianten van histatine 5 gemaakt. Deze varianten werden initieel aangemaakt door middel van bacteriële expressie, later werden ze synthetisch geproduceerd. Zo werd met varianten van histatine 5 aangetoond dat de fungicidale activiteit van histatine 5 ligt in een regio van residu 11 tot residu 24 aan de C-terminus van het peptide, dit is het zogenaamde functionele domein (Driscoll *et al.*, 1995) of dh-5 (Helmerhorst *et al.*, 1997). Mutaties in histatine 5 werden gecreëerd. Zowel enkelvoudige als meervoudige mutaties werden aangebracht, en de eigenschappen van deze peptiden werden bestudeerd. Zo werd aangetoond dat de mogelijkheid van histatine 5 om een α-helix te vormen niet essentieel is voor zijn antifungale eigenschappen, door een histatine variant te maken waarin drie aminozuren vervangen waren door prolines (Situ *et al.*, 2000). Deze variant (3P genoemd) bleek een gelijkaardige antifungale activiteit te bezitten als histatine 5, hoewel beperkt in zijn mogelijkheid om een α-helicale structuur te vormen. Histatine varianten waarbij de zure en basische aminozuren zijn vervangen in het functionele domein vertoonden een sterk verlaagde antifungale activiteit. Indien de histidines worden vervangen bleek de antifungale activiteit volledig verloren te gaan (Driscoll *et al.*, 1996). Indien Lys-13 vervangen werd door Thr (variant M21) of door Glu (variant M71) bleek dit ten koste te gaan van de antifungale activiteit (Tsai *et al.*, 1996). Twee synthetische peptiden met meerdere substituties (dhvar1 en dhvar2) bleken een verhoogde antifungale activiteit te bezitten. Naast de candidacidale activiteit bezitten deze peptiden ook een bactericidale activiteit tegen *Prevotela intermedia*, *Streptococcus mutans* en methicilline-resistente *Staphylococcus aureus* (Helmerhorst *et al.*, 1997). De mogelijkheid om deze peptiden als therapeutica te gebruiken werd onderzocht, maar deze weg werd al snel verlaten nadat bleek dat deze peptiden in vivo haemolytische activiteit vertoonden (Helmerhorst *et al.*, 1997).

Verschillende eigenschappen van histatine 5 hebben ertoe bijgedragen dat dit peptide sterk in de belangstelling staat als therapeuticum voor de behandeling van fungale infecties in de mond. Zo is er zijn sterke antifungale activiteit, verder is het een natuurlijk voorkomend peptide bij mensen en heeft het een andere manier van werken vergeleken met de azolen en polyenen (Tsai & Bobek, 1997), wat een voordeel is wanneer resistentie voor conventionele antifungale geneesmiddelen een probleem is (White *et al.*, 1998). P-113, een synthetisch histatine variant van 12 aminozuren lang (AKRHHGYKRKFH) bleek actiever dan histatine 5 en is onder andere actief tegen *Candida albicans*, *Candida glabrata, Candida parapsilosis* and *Candida tropicalis* (Rothstein *et al.*, 2001). In preklinische studies bleek dat uitwendig toegediend P-113 experimentele gingivitis verhindert bij honden. Honden behandeld met dit peptide vertoonden een significante vermindering van tandaanslag, gingivitis en bloedingen van het tandvlees in vergelijking met honden uit de placebo groep (Paquette *et al.*, 1997). Na deze succesvolle studie werden klinische studies opgestart om de veiligheid en effectiviteit van P-113 in mondspoeling

of topicale gel na te gaan. Uit deze studies bleek opnieuw dat P-113 in staat is om tandaanslag, gingivitis en bloedingen van het tandvlees te verminderen. Belangrijk is bovendien dat er geen nadelige symptomen of een nadelige verschuiving in de orale microflora aangetoond werd na het gebruik van mondspoeling of gel met P-113, wat aantoont dat het gebruik ervan in de mond veilig is voor mensen (Mickels *et al.*, 2001; Paquette *et al.*, 2002; Van Dyke *et al.*, 2002).

2 *Candida albicans*

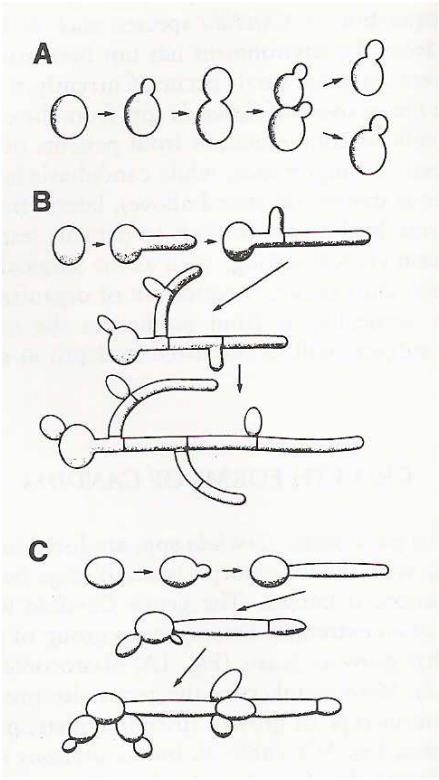
In dit tweede hoofdstuk van de literatuurstudie zal het organisme dat het onderwerp is van deze thesis, namelijk *Candida albicans*, kort besproken worden. Er zal een algemeen beeld geschetst worden van deze gist en bepaalde eigenschappen/karakteristieken zullen meer in detail behandeld worden.

2.1 Celbiologie

De meeste fungale infecties die bij mensen voorkomen zijn opportunistische infecties, aangezien het infecterende organisme meestal ofwel voorkomt als commensaal in het gastheerorganisme ofwel omdat het in grote getale aanwezig is in het leefmilieu. Ook *Candida albicans* leeft als ongevaarlijke commensaal in de mondholte, het spijsverteringsstelsel of de vagina. *C. albicans* kan echter infecties veroorzaken, die gaan van relatief onschuldige topicale infecties tot systemische infecties, wat kan resulteren in een fatale afloop. De laatste jaren is het aantal opportunistische infecties alsook de mortaliteit als gevolg van deze infecties sterk gestegen (Groll *et al.*, 1998). Dit is te wijten aan verschillende factoren (Fox, 1993): de uitbreiding van het aantal ernstig zieke of immuun-gecompromitteerde personen (borelingen met een extreem laag geboortegewicht, mensen geïnfecteerd met HIV, patiënten die zware chemotherapie ondergaan, immuunsuppressie na transplantatie, mensen met zware brandwonden, ...), de stijging in chirurgische ingrepen, de toename in gebruik van corticosteroïden en breed-spectrum antibiotica. Een groot aantal antifungale middelen zijn gekend (Polak, 1997), maar deze zijn echter beperkt bruikbaar als therapeuticum wegens hun toxische bijwerkingen (Georgopapadakou & Walsh, 1996). Enkele antifungale therapeutica zijn momenteel voorhanden en slechts sporadisch komen er nieuwe bij. De reden voor deze trage ontwikkelingen is het feit dat zowel fungale als zoogdiercellen, eukaryote cellen zijn. Producten die de fungale eiwit-, DNA- of RNA-synthese inhiberen zullen in veel gevallen ook toxisch zijn voor zoogdiercellen.

Een groot deel van de fungale infecties wordt veroorzaakt door *Candida* species, met *C. albicans* als meest voorkomende (Beck-Sague & Jarvis, 1993). Het genus *Candida* bestaat uit een extreem heterogene groep van organismen die groeien als gist, dit wil zeggen dat ze hoofdzakelijk als unicellulairen voorkomen. De meeste leden van dit genus kunnen bovendien ook groeien als een filamenteuze vorm (pseudohyfen, pseudomycelium), maar *C. albicans* en *C. dubliniensis* kunnen ook echte hyfen vormen (mycelium). Het genus omvat tussen de 150 en 200 species (Odds, 1987) die biologisch zeer divers zijn en onderling gedifferentieerd worden door middel van koloniemorfologie, gebruik van koolstrofbron en fermentatie. Tot deze genus behoren gisten met affiniteit voor ascomyceten zowel als voor basidiomyceten.

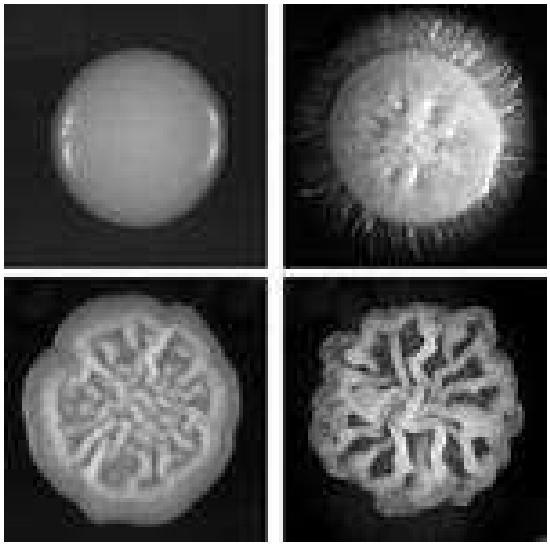
C. albicans is polymorf, het organisme groeit zowel als een knopvormende gistcel als in de vorm van een mycelium met de vorming van hyfen en pseudohyfen (Fig. 8). Het onderscheid tussen hyfen en pseudohyfen wordt gemaakt door hun ontstaanswijze. Pseudohyfen worden gevormd door een keten van geëlongeerde knoppen (blastoconidia) en worden gekarakteriseerd door een vernauwing bij cel-cel overgangen. Hyfen worden gekarakteriseerd door de aanwezigheid van parallelle wanden op de plaats waar de hyfe begint te ontwikkelen, de vorming van septa en de afwezigheid van vernauwingen.



Figuur 8: Voorstelling van de groei van *C. albicans* als gist (A), hyfen (B) en pseudohyfen (C) (Odds, 1988).

Hyfevorming wordt geïnduceerd door hoge temperatuur, een hoge CO_2/O_2 verhouding, neutrale pH en medium arm aan voedingsstoffen. Groei als unicellulaire gistvorm wordt daarentegen geïnduceerd door lage temperatuur, goede aeratie, zure pH en rijk medium (Soll, 1986). Deze gist-hyfe transities zijn geassocieerd met virulentie (Lo *et al.*, 1997).

Naast de mogelijkheid om te veranderen van groeivorm, van gist naar (pseudo)mycelium en omgekeerd, is *C. albicans* ook in staat om te veranderen van koloniemorfologie (Fig. 9). Deze hoog-frequentie fenotypeveranderingen leiden tot kolonievarianten die op hun beurt met een hoge frequentie opnieuw kunnen veranderen naar het originele fenotype.



Figuur 9: Vier fenotypes geïsoleerd uit een HIV patiënt (Vargas *et al.*, 2004).

De frequentie van deze spontane veranderingen kan variëren van 10^{-4} tot 10^{-1} (één verandering per 10^{4} tot 10^{1} celdelingen), en de verschillende fenotypes van een stam kunnen verschillende veranderingssnelheden vertonen (Slutsky *et al.*, 1985; Ramsey *et al.*, 1994). De frequentie van fenotypeverandering wordt beïnvloed door stresscondities zoals hitte, UV-radiatie en veroudering (Slutsky *et al.*, 1985; Rikkerink *et al.*, 1988; Morrow *et al.*, 1989; Soll, 1992). Alhoewel het mechanisme niet gekend is, zijn er aanwijzingen dat acetylatie/deacetylatie en *gene silencing* door de *SIR* genen een rol spelen in de erfelijke maar omkeerbare veranderingen van het chromatine nabij specifieke fenotypeverandering-loci (Perez-Martin *et al.*, 1999; Klar *et al.*, 2001; Srikantha *et al.*, 2001). Een verandering van fenotype beïnvloedt een aantal virulentiefactoren, waaronder de gist-hyfe transitie (Anderson *et al.*, 1989), bovendien bezitten verschillende fenotypes, een verschillende fitheid (Vargas *et al.*, 2004).

C. albicans is een diploid organisme met een genoom van 16 miljoen baseparen groot (haploid), ongeveer 30% groter dan dat van *S. cerevisiae*. Aan de hand van *pulsed-field* elektroforese werden 8 paar chromosomen geïdentificeerd, die genummerd worden van 1 (grootste) tot 7 (kleinste). Het chromosoom dat het ribosomaal DNA draagt wordt R genoemd. Dit R chromosoom kan sterk variëren in grootte, afhankelijk van het aantal rDNA herhalingen. Hierdoor kan het, naargelang zijn grootte, verschillende plaatsen innemen bij *pulsed-field* scheidingen en krijgt het de letter R toegewezen in plaats van een getal.

In 1996 begon het *Stanford Genome Technology Center* de sequenering van het *C. albicans* genoom (http://www-sequence.stanford.edu/group/candida/). De stam die werd gebruikt werd is *C. albicans* SC5314. De sequenering werd financieel gesteund door het *Burroughs Wellcome Fund*, het *National Institutes of Health* en het *National Institute for Craniofacial and Dental Research*. In 2004 werd het diploïde genoom gepubliceerd (Jones *et al.*, 2004).

Vele klinische isolaten vertonen karyotypes die niet overeen komen met de standaard. Deze karyotypische variabiliteit is te verklaren door de frequente chromosomale reorganisatie en recombinatie van het *C. albicans* genoom (Merz *et al.*, 1988). De reden voor de karyotypische variatie is onbekend, maar de modificaties kunnen toegeschreven worden aan selectie van bepaalde celpopulaties in verschillende condities, zoals nieuwe koolstofbronnen, aanwezigheid van antifungale stoffen of verschillende lichaamsniches. Sommige karyotypische veranderingen kunnen echter niet verklaard worden en lijken spontaan te ontstaan. Het is opvallend dat laboratoriumstammen relatief stabiele karyotypes vertonen (behalve voor chromosoom R).

De belangrijkste chromosomale veranderingen zijn het verschil in grootte van het R chromosoom en de recombinatie tussen niet-homologe chromosomen. De variatie in de grootte van het R chromosoom kan waarschijnlijk verklaard worden door de ongelijke *crossing-over* tussen homologen in het rDNA. Men heeft aangetoond dat 87 % van de spontane *C. albicans* mutanten een verandering in het R chromosoom vertonen (Rustchenko *et al.*, 1993) en dat 10 % van de nakomelingen van een cel de elektroforetische mobiliteit van dit chromosoom kunnen veranderen na 15 generaties als gevolg van variaties in de rDNA herhalingen (Iwaguchi *et al.*, 1992b).

Verder zijn nog drie evenementen geïdentificeerd die bijdragen tot de karyotypische variatie: aneuploïdie, translocaties en chromosomale lengte polymorfisme. Zowel translocaties als chromosomale lengte polymorfismen gebeuren ter hoogte van MRS (*Major Repeat Sequence*) gebieden. De MRS zijn herhalingssequenties, van tenminste 16 kbp lang, die voorkomen op alle chromosomen, behalve op chromosoom 3 (Chibana *et al.*, 1998; Chindamporn *et al.*, 1998). Niet-homologe chromosomen paren via de homologie van deze herhalingen en ondergaan vervolgens *crossing-over*. Lengte polymorfismen zijn te wijten aan het aantal kopieën van het 2 kbp centrale element van de MRS. Deze sequentie, RPS (*RePeated Sequence*) (Iwaguchi *et al.*, 1992a), kan tot meer dan vijfentwintig maal herhaald worden en wordt geflankeerd door twee geconserveerde regio's, HOK en RB2 (Chindamporn *et al.*, 1998). Chromosoom homologen van verschillende lengte blijken te verschillen in het aantal RPS in een of meerdere MRS gebieden (De Backer, 2000). De functionele significantie van translocaties en lengte polymorfismen bleef lang onduidelijk, tot Lephart *et al.* (2005) aantoonden dat de MRS een invloed hebben op de chromosoomstabiliteit.

2.2 Virulentiefactoren

Candida albicans veroorzaakt ongeveer 50 % van alle systemische schimmelinfecties en is in 90 % van de gevallen verantwoordelijk voor vaginale candidosen. Bovendien wordt het organisme gerekend tot de vierde meest frequente verwekker van sepsis in de Verenigde Staten (Pfaller *et al.*, 1998). Om een infectie te veroorzaken moeten opportunistische pathogenen het immuunsysteem ontwijken, overleven, delen in de gastheer en zich verspreiden naar andere

weefsels. *C. albicans* kan verschillende plaatsen in het lichaam, zoals de huid, de mondholte, de slokdarm, het gastrointestinaal stelsel, de vagina en het vasculair systeem koloniseren en daar een infectie veroorzaken. Deze plaatsen hebben dikwijls een unieke fysiologische omgeving. De karakteristieken die toelaten dat deze plaatsen geïnfecteerd geraken door *C. albicans* worden virulentiefactoren genoemd. In tegenstelling tot gespecialiseerde pathogenen die één enkele virulentiefactor expresseren (bv. *Clostridium tetani*), bezit de opportunistische pathogeen *C. albicans* verschillende eigenschappen die bijdragen aan de virulentie. De belangrijkste en best onderzochte virulentiefactoren zijn adhesie aan gastheercellen, gist-hyfe transitie en de productie van hydrolytische enzymen.

Herkenning van en adhesie aan de gastheercel van *C. albicans* is een essentiële vroege stap in het veroorzaken van een infectie en dus een belangrijke virulentiefactor. De componenten die verantwoordelijk zijn voor gastheerherkenning en kolonisatie worden adhesines genoemd (Calderone & Gow, 2002). Enkele adhesines die beschreven werden zijn, de Als (*Agglutenin-like sequence*) eiwit familie (Hoyer, 2001), Hwp1 (Staab *et al.*, 1999), Int1 (Kinneberg *et al.*, 1999), Mnt1 (Buurman *et al.*, 1998), Pmt1 (Timpel *et al.*, 1998) en Pmt6 (Timpel *et al.*, 2000). Verder blijkt ook de relatieve hydrofobiciteit van het oppervlak van *C. albicans* een invloed te hebben op de efficiëntie van aspecifiek vasthechten op oppervlakken (Calderone & Gow, 2002).

De mogelijkheid van *C. albicans* om om te schakelen van gistvorm naar filamenteuze vorm en omgekeerd is ook belangrijk voor zijn virulentie (Kumamoto & Vinces, 2005). Dit werd aangetoond door het gebruik van deletiestammen die deficiënt zijn in filamenteuze groei. Zo bleken de *cph1*/*cph1 efg1*/*efg1* dubbele deletiestam (Lo *et al.*, 1997) en de *crk1*/*crk1* en de *hgc1*/*hgc1* deletiestammen (Chen *et al.*, 2000; Zheng & Wang, 2004), stammen die geen hyfen kunnen vormen, avirulent te zijn in een muismodel. Tup1 is een negatieve regulator van filamenteuze groei, aangezien de *tup1*/*tup1* deletiestam constitutieve filamenteuze groei vertoont. Deze hyperfilamenteuze stam bleek ook een verminderde virulentie te bezitten (Braun & Johnson, 1997). Hieruit blijkt dat alleen stammen die hyfen en gistcellen kunnen produceren in staat zijn om vitale organen te penetreren en daar voldoende kunnen groeien om het gastheerorganisme te doden.

Een andere factor die bijdraagt tot virulentie van een microorganisme, is de productie van hydrolytische enzymen. Gesecreteerde hydrolytische enzymen kunnen bijdragen tot de invasie in gastheerweefsel door afbraak of verstoring van membranen, afbraak van oppervlakmoleculen van gastheercellen om adhesie te vergemakkelijken, of afbraak van cellen of moleculen van het gastheer immuunsysteem (Hube & Naglik, 2002). *C. albicans* produceert hydrolytische enzymen met brede substraatspecificiteit. De drie meest belangrijke hydrolytische enzymen in *C. albicans* zijn gesecreteerde aspartyl proteasen (SAP) (Naglik *et al.*, 2003), fosfolipase B enzymen en lipasen (Ghannoum, 2000; Hube & Naglik, 2002). De Sap proteïnen, gecodeerd

door een familie van 10 *SAP* genen, zijn van deze drie het meest bestudeerd (Naglik *et al.*, 2003).

Deze drie eigenschappen zijn niet de enige die bijdragen tot de virulentie van *C. albicans*. Andere virulentiefactoren die beschreven zijn, zijn de hoge-frequentie fenotypeveranderingen (Yang, 2003), thigmotropisme (d.i. de mogelijkheid om contact te voelen), moleculaire mimicry, groeisnelheid en de mogelijkheid om te groeien in een milieu met weinig nutriënten (Cutler, 1991; Odds, 1994).

Geen enkele *C. albicans* virulentiefactor op zich is echter verantwoordelijk voor virulentie en niet al de geëxpresseerde virulentiefactoren zijn noodzakelijk voor een bepaalde fase of plaats van infectie.

2.3 Mating

Sinds zijn moderne klassificatie zo'n 100 jaar geleden is *Candida albicans* altijd omschreven als een asexuele gist. In de jaren '70 en '80 van vorige eeuw werd aangetoond dat natuurlijke isolaten van *C. albicans* diploid zijn, geen enkele observatie suggereerde de aanwezigheid van een sexuele reproductieve cyclus (Olaiya & Sogin, 1979; Whelan *et al.*, 1980). Dit was verrassend, gezien de dichte relatie van *C. albicans* met *Saccharomyces cerevisiae* en *Kluyveromyces lactis*, gisten die wel sexueel reproduceren. Tegen het einde van vorige eeuw echter werden van verschillende *S. cerevisiae* genen, waarvan aangetoond was dat ze betrokken zijn bij *mating* en meiose, orthologen ontdekt in *C. albicans*. Sommige van deze *C. albicans* orthologen konden bovendien hun tegenhangers complementeren indien ze geexpresseerd werden in *S. cerevisiae* (Diener & Fink, 1996; Leberer *et al.*, 1996). Met de sequenering van het volledige genoom van *C. albicans* konden Hull en Johnson twee *mating-type* loci, *MTL*a en *MTL*α, identificeren (Hull & Johnson, 1999).

Zowel *MTL*a als *MTL*α bevatten twee functionele genen, a1 en a2 voor *MTL*a en α1 en α2 voor *MTL*α (Tsong *et al.*, 2003). Opdat fungi sexueel zouden kunnen reproduceren is functionele hemizygotie of homozygotie van de *mating* locus vereist, dat terwijl de meeste *C. albicans* isolaten heterozygoot zijn. Toch werd binnen het jaar *mating* aangetoond door twee groepen. Door deletie maakten Hull en collega's stammen hemizygoot voor *MTL*a of α en zij toonden mating aan in muizen door het injecteren van een gemengde cultuur (Hull *et al.*, 2000). Magee en Magee maakten homozygote stammen door chromosoom verlies, geïnduceerd door groei op sorbose, en toonden *mating* aan op plaat (Magee & Magee, 2000).

Miller en Johnson toonden aan dat homozygote *MTL*a en *MTL*α stammen, gemaakt van de CAI-4 stam, wit-opaak transitie ondergaan en dat *mating* 10^6 keer efficiënter gebeurt indien beide stammen in de opaak fase zijn. De mogelijkheid van een *C. albicans* stam om wit-opaak transitie te ondergaan blijkt gereguleerd te worden door de *MTL* locus (Miller & Johnson, 2002). Deze bevindingen verklaren waarom slechts 3 tot 7% van de klinische isolaten van *C.*

albicans in staat is om de wit-opaak transitie te ondergaan: a en α cellen wel, maar a/α cellen niet. *Mating* van *C. albicans* cellen is dus sterk verbonden met de wit-opaak transitie. Waarschijnlijk kunnen alleen opaak cellen zich sexueel reproduceren, de lage frequentie van *mating* die gebeurt tussen witte cellen is waarschijnlijk een gevolg van het spontaan ontstaan van opaak cellen (Bennett & Johnson, 2005).

Het *mating* proces in *C. albicans* kan in verschillende stappen ingedeeld worden en komt in grote mate overeen met het *mating* proces in *S. cerevisiae*, alhoewel er toch enkele belangrijke verschillen zijn (Magee *et al.*, 2002). Indien diploïde opaak a en α cellen gemengd worden dan vormen ze *mating* projecties die, in tegenstelling met de *shmoo*-vormen in *S. cerevisiae*, tot verschillende malen de lengte van de cel groeien. Het uiteindelijke resultaat van het *mating* proces zijn tetraploïde cellen. In de meeste fungi is er een meiose proces aanwezig om terug naar diploïde cellen te gaan. Alhoewel er in *C. albicans* orthologen zijn geïdentificeerd van genen van andere fungi die betrokken zijn bij meiose, is in *C. albicans* dit meiose proces nog niet beschreven (Tzung *et al.*, 2001). In sommige fungi gebeurt reductie van ploïdie door chromosoomverlies gedurende opeenvolgende mitotische delingen om zo een parasexuele cyclus te vormen. Alhoewel er nog geen meiotische cyclus is geïdentificeerd, toch is er een parasexuele cyclus beschreven met efficient chromosoom verlies (Bennett & Johnson, 2003).

2.4 Codongebruik

In de jaren 1960 werd door een combinatie van *in vitro* en *in vivo* experimenten duidelijk dat, ondanks een hoge graad van redundantie binnen de genetische code (het gebruik van meer dan één codon voor een specifiek aminozuur), deze genetische code universeel bleek te zijn (Crick, 1968). Bijgevolg stelde Crick dat "*the (genetic) code is universal because at the present time change would be lethal, or at least very strongly selected against*" (Crick, 1968). Deze stelling bleef geaccepteerd tot een aantal onverwachte afwijkingen de kop op staken in het begin van de jaren '80. Er bestaan voorbeelden waar volledige omvorming gebeurde van *non-sense* codons tot *sense* codons en omgekeerd. Omvormingen van *sense* naar *sense* codons zijn veel zeldzamer, met één zeer goed gedocumenteerd geval, namelijk de toewijzing van het CUG codon van leucine naar serine in veel *Candida* species (O'Sullivan *et al.*, 2002).

Een eerste tabel voor codon gebruik in *C. albicans* werd gepubliceerd door Brown *et al.* (1991), deze tabel werd opgesteld door het gebruik van codons na te gaan van slechts een beperkt aantal coderende sequenties. *C. albicans* bleek een sterke voorkeur te hebben voor het gebruik van een A of T op de derde plaats in het codon, de zogenaamde Wobble-base. Lloyd en Sharp (Lloyd & Sharp, 1992) verrichtten een meer grondige analyse van het codongebruik in *C. albicans* en probeerden de optimale codons voor dit organisme te identificeren. Ook zij vonden een sterke voorkeur voor het gebruik van A of T op de derde positie van de codons. Bovendien, vonden zij dat het gebruik van A of T op de eerste plaats van de codons vermeden wordt.

De belangrijkste afwijking op de universele genetische code in *C. albicans* is het gebruik van het CUG codon voor serine in plaats van leucine. Deze verandering in het gebruik van de genetische code is niet algemeen in het genus *Candida*. Naast *C. albicans* komt deze wijziging voor in 75 leden van het genus. In sommige species wordt het CUG codon zowel voor serine als leucine gebruikt (*C. albicans, C. maltosa, C. zeylanoides, C. lusitaniae* en *C. tropicalis*). In de meeste species wordt het CUG codon echter alleen gebruikt voor serine. In de species waar het CUG codon voor beide aminozuren wordt gebruikt, blijkt echter het gebruik voor leucine echter minimaal. In *C. zeylanoides,* bijvoorbeeld, wordt in vivo slechts 3% van het Ser-$tRNA_{CAG}$ geleucyleerd, terwijl het voor de rest wordt geseryleerd en bijgevolg de incorporatie in polypeptiden gebeurt volgens deze verhouding (Suzuki *et al.,* 1997).

De meest gebruikte leucine codons in *C. albicans* zijn UUA en UUG terwijl de CUN codon familie zelden gebruikt wordt. In deze familie komt het CUU codon het meest voor, gevolgd door de CUA en CUC codons. De lage frequentie van het gebruik van het CUG codon, dat codeert voor serine, komt overeen met het weinig voorkomen van het seryl-$tRNA_{CAG}$. Het CUG codon wordt vooral gebruikt in genen die een laag expressieniveau bezitten (Santos & Tuite, 1995).

Dit niet-universeel codon gebruik van *C. albicans* heeft gevolgen voor de heterologe expressie van genen in dit organisme. Heterologe genen die CUG codons bevatten zullen niet correct geëxpresserd worden in *C. albicans.* Alle CUG codons moeten daarom vervangen worden door de leucine codons UUA of UUG. Dit is bijvoorbeeld gedaan om het *green fluorescent protein* (yeGFP) rapporteergen geëxpresseerd te krijgen in *C. albicans* (Cormack *et al.,* 1997).

3 Bax-geïnduceerde celdood in gist

3.1 De Bcl-2 familie

Genetisch geprogrammeerde celdood (apoptose) is uitgebreid beschreven en onderzocht in multicellulaire eukaryoten. Overbodige, ongewenste of potentieel schadelijke cellen voor het organisme ondergaan een apoptotisch proces dat gekarakteriseerd wordt door het krimpen van de cel, chromatine- en cytoplasmacondensatie, degradatie van nucleair DNA, plasmamembraan *blebbing* en fagocytose van het celdebris (Schwartz *et al.*, 1993). De Bcl-2 familie van eiwitten speelt een centrale rol in de controle van dit geprogrammeerde celdoodproces. Deze familie van eiwitten bestaat uit verschillende subfamilies. De Bcl-2 subfamilie bevat eiwitten met anti-apoptotische activiteit en bestaat uit Bcl-2, Bcl-X_L, Bfl1/A1, Bclw en BclB. De Bax subfamilie bevat eiwitten met pro-apoptotische activiteit en omvat onder andere Bax, Bak en Bok. De Bik subfamilie heeft een indirecte activiteit en leden van deze subfamilie kunnen over het algemeen als pro-apoptotisch beschouwd worden door het feit dat ze leden van de anti-apoptotische Bcl-2 familie direct inactiveren, pro-apoptotische eiwitten vrijstellen van hun anti-apoptotische tegenpolen, of pro-apoptotische eiwitten direct activeren. Verschillende geconserveerde domeinen werden geïdentificeerd in de Bcl-2 familie, aangeduidt als Bcl-2 Homologie Domeinen, BH1, BH2, BH3 en BH4. Alle vier de homologie domeinen kunnen teruggevonden worden in de Bcl-2 subfamilie, de Bax subfamilie mist over het algemeen BH4 en de Bik subfamilie bezit enkel het BH3 domein (uitgezonderd Bcl-X_S, dat ook BH4 bezit). De meeste leden van deze familie bezitten verder nog een hydrofoob carboxy-terminaal fragment, het TransMembraan (TM) domein. Dit domein zou de interactie met intracellulaire membranen verzorgen (Reekmans, 2004).

De proteïnen van de Bcl-2 familie zijn multifunctioneel. Zij kunnen dimeriseren met andere Bcl-2 familieleden, zij beschikken over een porie-vormende of ionkanaal-vormende activiteit en zij kunnen binden aan niet-homologe eiwitten (Zamzami *et al.*, 1998). De verhouding tussen pro- en anti-apoptotische Bcl-2 eiwitten bepaalt de gevoeligheid van een cel voor apoptose, het zogenaamde rheostaat model. Deze hypothese postuleert dat er een delicate balans bestaat tussen pro- en anti-apoptotische Bcl-2 eiwitten, een verandering in deze verhouding bepaalt het lot van de cel: overleven of sterven. Het anti-apoptotische Bcl-2 is in staat om heterodimeren te vormen met het pro-apoptotische Bax. Bcl-2 kan op deze manier voor celoverleving zorgen door Bax te complexeren en op deze manier de apoptotische activiteit van Bax te neutraliseren (Korsmeyer *et al.*, 1993; Oltvai *et al.*, 1993). Naast deze heterodimerisatie capaciteit is ook aangetoond dat Bax membranen kan destabiliseren (Basanez *et al.*, 1999) en kan insereren in membranen met de vorming van ionkanalen (Antonsson *et al.*, 1997). De interactie van Bax met membranaire dubbellagen zorgt voor de vorming van poriën die groot genoeg zijn om eiwitten te laten passeren zoals cytochroom c (Saito *et al.*, 2000; Epand *et al.*, 2002). Complexatie van

Bcl-2 met Bax zou deze porie-vorming verhinderen (Antonsson *et al.*, 1997). Bovendien zou Bax kunnen binden met de *Voltage-Dependent Anion Channel* (VDAC) (Narita *et al.*, 1998) en de *Adenine-Nucleotide Transporter* (ANT) (Marzo *et al.*, 1998), respectievelijk gelegen in de buitenste en binnenste mitochondriale membraan.

Bax-expressie op zich induceert in zoogdiercellen geen apoptose (Deckwerth *et al.*, 1996), overexpressie zorgt wel voor een versnelde celdood na een celdood signaal (Oltvai *et al.*, 1993) en hyperexpressie van Bax is voldoende om celdood te induceren zonder een stimulus (Xiang *et al.*, 1996). Apoptose is geassocieerd met een herlokalisatie van de Bax eiwitten van het cytosol naar de mitochondriën (Wolter *et al.*, 1997), ER (Zong *et al.*, 2003), Golgi (Godlewski *et al.*, 2001) en nucleaire membranen (Mandal *et al.*, 1998). Verder zijn verschillende pro-apoptotische effecten geassocieerd met de overexpressie van Bax in zoogdiercellen in celcultuur: modulatie van celcyclus, depletie van Ca^{2+} in het ER, accumulatie van Ca^{2+} in de mitochondriën, accumulatie van eiwitten in het ER en de mitochondriën, cytochroom c vrijstelling uit de mitochondriën, mitochondriale fragmentatie, aggregatie van de mitochondriën rond de kern, een verhoging van de ROS productie, een verlaging of verlies van de mitochondriale membraanpotentiaal en een verlaging in gluthation (Reekmans, 2004).

Expressie van Bax induceert niet alleen celdood in zoogdiercellen, heterologe expressie van Bax veroorzaakt ook celdood in *Drosophila melanogaster* (fruitvlieg) (Gaumer *et al.*, 2000), *Nicotiniana benthaminiana* (tabaksplant) (Lacomme & Santa Cruz, 1999), *Arabidopsis thaliana* (zandraket) (Kawai-Yamada *et al.*, 2001), de bacterie *Escherichia coli* (Asoh *et al.*, 1998), en in de gisten *Saccharomyces cerevisiae* (Sato *et al.*, 1994; Greenhalf *et al.*, 1996), *Schizosaccharomyces pombe* (Jurgensmeier *et al.*, 1997), *Pichia pastoris* (Martinet *et al.*, 1999) en *Kluyveromyces lactis* (Poliakova *et al.*, 2002).

3.2 Bax-geïnduceerde celdood in gist

Eén van de karakteristieke eigenschappen van geprogrammeerde celdood en apoptose is de autonome manier waarop het celdoodproces in een cel gebeurt, de stervende cellen zijn actieve deelnemers in hun eigen celdood (Ucker, 1991). Ook in unicellulaire organismen zijn deze zelfmoordprocessen herkend, wat wijst op een mogelijke evolutionaire oorsprong (Shub, 1994). Op basis van de bevindingen dat Bax celdood kan induceren in verschillende gisten en dat deze celdoodprocessen verhinderd kunnen worden door de co-expressie van Bcl-X_L of Bcl-2, wordt de mogelijkheid geopperd dat ten minste een deel van het Bcl-2 familie gecontroleerde celdood proces evolutionair geconserveerd is. De studie van Bax-geïnduceerde celdood in gist kan dus bijdragen aan het ophelderen van een evolutionair geconserveerd celdoodmechanisme.

Heterologe overexpressie van het pro-apoptotische eiwit Bax in *S. cerevisiae* cellen induceert celdood in deze gistcellen (Greenhalf *et al.*, 1996). Dit celdoodproces werd initieel beschreven

als gelijkend op autofagie met de desintegratie van de organellen en vacuolisatie. Apoptotische veranderingen, zoals morfologische veranderingen van de kern, compacte chromatine condensatie en oligonucleosomale DNA degradatie, werden aanvankelijk niet waargenomen (Zha *et al.*, 1996). Daarom werd gesuggereerd dat Bax-geïnduceerd celdood in bakkersgist enkel te wijten zou zijn aan de intrinsieke toxiciteit van het eiwit zelf, als gevolg van zijn porie-vormende eigenschappen (Muchmore *et al.*, 1996). Meer recenter werk toonde echter verschillende morfologische veranderingen aan in gistcellen die Bax expresseren, die overeenkomen met reeds eerder geobserveerde veranderingen in apoptotische zoogdiercellen. Ligr *et al.* (1998) toonden aan dat heterologe overexpressie van Bax in *S. cerevisiae* morfologische veranderingen veroorzaakt, zoals losse chromatine condensatie, mitochondriale zwelling, breuken in DNA-strengen en plasmamembraan *blebbing*.

Net zoals in zoogdiercellen lokaliseert Bax in bakkersgist naar de mitochondriën (Wolter *et al.*, 1997), insereert Bax in de mitochondriale membranen (Priault *et al.*, 1999), zorgt voor cytochroom c vrijstelling (Manon *et al.*, 1997) en induceert de productie van *reactive oxygen species* (ROS) (Madeo *et al.*, 1999). De expressie van Bax veroorzaakt schade aan de mitochondriën (Priault *et al.*, 2002) wat leidt tot verlies van mitochondriale functies (Kissova *et al.*, 2000), waarschijnlijk als gevolg van de vorming van een porie in de buitenste mitochondriale membraan (Epand *et al.*, 2002).

Jürgensmeier *et al.* (1997) gingen de gevolgen van Bax-expressie in *Schizosaccharomyces pombe* na en vergeleken dit met Bax-geïnduceerde celdood in zoogdiercellen. Verschillende observaties toonden gelijkenissen aan tussen de effecten van dit eiwit in deze gist en zoogdiercellen. Bax-expressie in *S. pombe* resulteerde in celdood. Dit lethale effect werd teniet gedaan daar de coëxpressie van de anti-apoptotische eiwitten Bcl-X_L of Bcl-2. Een mutant van Bcl-2, die het pro-apoptotische effect van Bax in zoogdiercellen niet kan verhinderen (Yin *et al.*, 1994), is ook niet in staat om in *S. pombe* het celdood proces te verhinderen. Andere observaties daarentegen tonen aan dat er verschillen zijn in het celdoodfenotype tussen deze gist en zoogdiercellen na Bax expressie. Het Bax-geïnduceerde celdoodproces van *S. pombe* cellen gaat gepaard met cytoplasmatische vacuolisatie, cytosolische condensatie en multifocale nucleaire condensatie, maar niet met verkleining van het celvolume, nucleaire fragmentatie en protease activiteit. Verder bleek ook de protease inhibitor p35 niet in staat om Bax-geïnduceerde celdood in *S. pombe* te verhinderen (Jurgensmeier *et al.*, 1997).

Bax-expressie in de methylotrofe gist *P. pastoris* resulteerde in volledige groei-inhibitie en celdood. Behalve chromatine condensatie, werden geen morfologische veranderingen waargenomen die typisch zijn voor apoptose in zoogdiercellen. Kleuring van de Bax-expresserende cellen met propidium jodide toonde aan dat de waargenomen celdood niet gecorreleerd kan worden met necrose. Elektronenmicroscopische opnamen toonden enkele

cellen met kenmerken die een celdoodproces suggereren dat eerder gecorreleerd is met autofagie (Martinet *et al*., 1999).

Ook in *Kluyveromyces lactis* resulteerde de intracellulaire expressie van Bax in groei-inhibitie en celdood. Deze celdood ging gepaard met een verhoogde ROS productie. Het anti-apoptotisch eiwit Bcl-X_L, indien co-geëxpresseerd met Bax, localiseert naar de mitochondriën en verhindert Bax-geïnduceerde celdood maar niet de ROS productie. Respiratorisch deficiënte ρ^0 mutanten van *K. lactis* zijn gevoeliger voor Bax dan de wild type cellen, wat erop wijst dat Bax-geïnduceerde celdood in deze gist niet afhankelijk is van de functie van de mitochondriale elektronentransportketen (Poliakova *et al*., 2002).

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Deel II
DOELSTELLING

DOELSTELLING

De laatste twee decennia is er een sterke stijging van het aantal opportunistische fungale infecties geweest, ook de mortaliteit als gevolg van deze infecties is gestegen. Het organisme dat verantwoordelijk is voor de meeste van de fungale infecties is *Candida albicans*. Deze gist leeft als ongevaarlijke commensaal in mensen, waar het kan voorkomen in de mondholte, het spijsverteringsstelsel of de vagina. *C. albicans* kan echter infecties veroorzaken, die gaan van relatief onschuldige topicale infecties tot systemische infecties. In ongeveer 50% van de gevallen resulteren deze systemische infecties in een fatale afloop. Een groot aantal antifungale middelen zijn gekend, maar deze zijn echter beperkt bruikbaar als therapeuticum wegens hun toxische bijwerkingen. Slechts enkele antifungale therapeutica zijn momenteel voorhanden en slechts sporadisch komen er nieuwe bij. Bovendien treedt er resistentie op tegen de frequent gebruikte antifungale middelen. Daarom zijn er nieuwe antifungale middelen nodig die inwerken op nieuwe moleculaire doelwitten en werken volgens een verschillend mechanisme in vergelijking met de gangbare antifungale middelen.

Het doel van deze thesis is het induceren van een celdoodproces in *C. albicans* om daarna na te gaan welke genen betrokken zijn bij dit celdoodproces. De genen die betrokken zijn bij het celdoodproces kunnen gebruikt worden als doelwit voor de ontwikkeling van nieuwe antifungale middelen.

Er wordt geopteerd om op twee verschillende manieren een celdoodproces te initiëren. Een eerste manier is eerder een artificiële manier: door de conditionele intracellulaire expressie van het pro-apoptotische eiwit Bax proberen we een celdoodproces in gang te zetten. De intracellulaire expressie van dit eiwit in enkele andere gisten toonde aan dat het toxisch is voor de gistcel en celdood initieert. Bax is een eiwit dat niet voorkomt in gisten, het wordt geëxpresseerd in zoogdiercellen waar het een centrale rol speelt in de controle van geprogrammeerde celdood. Een andere manier die gebruikt zal worden om celdood te veroorzaken, is door de incubatie van de *C. albicans* cellen met het antimicrobiële peptide, histatine 5. Dit is een fysiologisch relevante manier om celdood te initiëren. Het histatine 5 is een peptide met sterke antifungale eigenschappen dat voorkomt in het speeksel van de mens en andere hogere primaten. Dit peptide maakt deel uit van het aangeboren immuunsysteem en is onder andere verantwoordelijk voor het voorkomen van infecties in de mondholte.

Om genen te identificeren die betrokken zijn bij het histatine 5-geïnduceerde celdoodproces zullen twee strategieën gevolgd worden. Aan de hand van de *screening* van een overexpressiebank zullen we genen kunnen identificeren die, indien ze overgeëxpresseerd worden, een meer resistent fenotype verlenen aan de cellen. Aan de hand van microarray analyse kunnen we het expressieprofiel bepalen dat samenvalt met histatine 5-geïnduceerde celdood. De meest interessante genen die met deze twee strategieën geïdentificeerd zullen worden, kunnen dan verder bestudeerd worden aan de hand van overexpressie- en deletiestudies

om hun betrokkenheid bij het histatine 5-geïnduceerde celdoodproces te bepalen. De genen die betrokken zijn bij dit celdoodproces kunnen gebruikt worden als doelwit voor de ontwikkeling van nieuwe antifungale middelen.

Naast de identificatie van genen die betrokken zouden kunnen zijn bij het celdoodproces in *C. albicans*, wordt het Bax-geïnduceerde en histatine 5-geïnduceerde celdoodproces ook verder bestudeerd in celbiologisch opzicht. Het histatine 5-geïnduceerde celdoodproces in *C. albicans* wordt verder ook vergeleken met het histatine 5-geïnduceerde celdoodproces in *Saccharomyces cerevisiae*, een gist die vaak als modelorganisme gebruikt wordt in het onderzoek naar *C. albicans.*

Deel III
RESULTATEN

1 Bax-geïnduceerde celdood in *Candida albicans*

In dit eerste hoofdstuk onderzoeken we de effecten van Bax-expressie in de pathogene gist *C. albicans*. De resultaten van dit werk werden gepubliceerd in *Yeast*.

Celdood inducerende expressie van het pro-apoptotische eiwit Bax kon verkregen worden door het gebruik van een synthetisch *BAX* gen waarvan de codons aangepast werden voor optimale expressie in *C. albicans*. Expressie van dit synthetische *BAX* gen resulteerde in groei-inhibitie en celdood. Door Bax te fuseren met het gist-geöptimaliseerd *green fluorescent protein* van *Aequoria victoria* werd het celdood inducerende effect van Bax verhoogd door een verminderde proteolytische degradatie van Bax. Door gebruik te maken van dit fusie-eiwit konden we aantonen dat Bax met de mitochondriën colokaliseert. Verder konden we voor de allereerste keer aantonen dat expressie van Bax in gist ervoor zorgt dat de mitochondriën, die normaal een verspreide distributie in de cel vertonen, groeperen in een regio nabij de kern.

Artikel

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Bax-induced cell death in *Candida albicans*

Kris De Smet, Ines Eberhardt, Rieka Reekmans and Roland Contreras*

Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, B-9052 Gent, Belgium

* Corresponding author. Mailing address: Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, B-9052 Gent, Belgium. Phone: ++32 9 331 3631. Fax: ++32 9 331 3502. E-mail: Roland.Contreras@dmbr.Ugent.be

Keywords: Candida albicans, cell death, Bax, Gfp, mitochondrial clustering

Abstract

Bax is a pro-apoptotic member of the Bcl-2 family of proteins involved in the regulation of genetically programmed cell death in mammalian cells. It has been shown that heterologous expression of Bax in several yeast species, such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Pichia pastoris*, also induces cell death. In this study we investigated the effects of Bax expression in the pathogenic yeast *Candida albicans*. Cell death inducing expression of Bax required a synthetic *BAX* gene that was codon-optimized for expression in *Candida albicans*. Expression of this *BAX* gene resulted in growth inhibition and cell death. By fusing Bax with the yeast enhanced Green Fluorescent Protein of *Aequoria victoria*, the cell death-inducing effect of Bax was increased due to reduced proteolytic degradation of Bax. Using this fusion protein we showed that, upon expression in *C. albicans*, Bax co-localizes with the mitochondria. Furthermore, we showed for the first time that expression of Bax in yeast causes the mitochondria, which are normally distributed throughout the cell, to cluster in the perinuclear region.

Introduction

In recent years there has been a spectacular increase in human fungal infections (Fox, 1993). Most of these infections are attributed to *Candida albicans*, a frequently encountered commensal in the oral cavity, vagina and digestive tract. *C. albicans* can cause not only topical infections, but also life-threatening systemic infections, especially in immunocompromised patients. As the number of effective non-toxic anti-fungal compounds is limited, and resistance to frequently used drugs is increasing, there is an urgent need for new antifungal drugs. In this paper we describe a new system for conditional cell death in *C. albicans*, based on intracellular expression of the pro-apoptotic protein Bax. This conditional cell death system can be used for the identification of new targets for cell death-modulating drugs.

Genetically programmed cell death (apoptosis) has been described in multicellular eukaryotes. Cells that are unwanted or potentially harmful to the organism undergo an apoptotic process characterized by cell shrinkage, chromatin and cytoplasmic condensation, digestion of nuclear DNA, loss of mitochondrial membrane potential, plasma membrane blebbing, and phagocytosis of the cell debris (Schwartz *et al.*, 1993). The Bcl-2 family of proteins is centrally involved in the control of the programmed cell death process (PCD). Proteins of this group belong either to the inhibitors of cell death (Bcl-2, Bcl-X_L) or to the group of proteins promoting apoptosis (Bax, Bak) (Oltvai & Korsmeyer, 1994; Knudson & Korsmeyer, 1997; Reed *et al.*, 1998).

Expression of the pro-apoptotic Bcl-2 family member Bax in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Pichia pastoris* induces cell death (Sato *et al.*, 1994; Greenhalf *et al.*, 1996; Zha *et al.*, 1996; Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997; Martinet *et al.*, 1999). In *S. cerevisiae* this process was initially described as resembling autophagy, with dissolution of the internal organelles and vacuolization. But apoptotic changes, such as morphological changes in nuclear shape and chromatin condensation, were not observed in this yeast (Zha *et al.*, 1996). It was therefore suggested that Bax-induced cell death in *S. cerevisiae* is due to the intrinsic toxicity of the Bax protein itself, mediated solely by pore-formation (Muchmore *et al.*, 1996). However, more recent work revealed a series of morphological changes in yeast cells expressing human Bax similar to those observed in apoptotic metazoan cells (Ligr *et al.*, 1998). Bax expression in the fission yeast *Schizosaccharomyces pombe* also showed some of the morphological changes typical of apoptosis, such as DNA fragmentation, chromatin condensation, dissolution of the nuclear envelope and cytosolic vacuolization, suggesting the presence of a PCD pathway in this unicellular eukaryote (Ink *et al.*, 1997; Jurgensmeier *et al.*, 1997). Bax expression in *P. pastoris* resulted in growth inhibition and cell death. Chromatin condensation, typical of apoptosis in mammalian cells, was also observed. Propidium iodide staining indicated that cell death was not correlated with necrosis (Martinet *et al.*, 1999).

Considering the observation of Bax induced cell death in *S. cerevisiae*, *S. pombe* and *P. pastoris*, a similar death regulating mechanism could also be present in other yeasts. We therefore asked whether overexpression of the mouse Bax protein leads to cell death in the pathogenic yeast *Candida albicans*. Functions involved in this Bax-induced cell death process can be used as targets for the development of novel antifungal compounds.

Methods

Strains

C. albicans CAI-4 (*ura3::imm434/ura3::imm434)* was kindly provided by Dr. W. Fonzi, Georgetown University (Washington, DC) (Fonzi & Irwin, 1993).

Escherichia coli transformations were done using the Top10 strain from Invitrogen (San Diego, CA) (F' *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G).

Media

Synthetic dextrose medium (SD), containing 2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids and 0.077% (w/v) complete supplement mixture minus uracil (BIO 101 Inc., Carlsbad, CA) was used to grow the *C. albicans* transformants. To ensure growth of the wild type *C. albicans* (CAI-4), the media was supplemented with 50 µg/ml uridine. For plate preparation the medium was solidified with 2% (w/v) agar. Expression of the synthetic *BAX* gene was performed using 2% (w/v) galactose as carbon source.

Construction of the codon-optimized BAX gene

Construction of the synthetic *BAX* gene followed the codon usage described for *C. albicans* (Brown *et al.*, 1991; Lloyd & Sharp, 1992) (http://alces.med.umn.edu/candida/codons.html). To ensure a high level of expression of the synthetic gene, the subset of 'optimal' codons of highly expressed genes was used to design the synthetic *BAX* gene.

The synth*CaBAX* gene was constructed in three parts using eight oligonucleotides. The sequences of the oligonucleotides are given in Table 1. Primer A1 introduced a *Pst*I site for direct cloning into the *C. albicans* expression vector, and a *Bgl*II site upstream of the ATG codon as a linker for a yE*GFP* fusion. Primer C2 introduced a *Sma*I site for cloning into the expression vector.

Table 1. Oligonucleotides used for construction of the synthetic *CaBax* gene.

Oligo	Sequence 5' to 3'
A1	AACTGCAGGAAGATCTTCC**ATG**GATGGTTCTGGTGAACAATTGGGTTCTGGTGGTCCAACCTCTTCTGAACAAATCATGAAAACCGGTGCTTTCTTGTTG
A2	TAGAAGCATCTTGTGGTGGTTGTTCCAAGGTCAATTCTGGGGTTTCACCAGCCATTCTACCAGCTCTATCTTGGATGAAACCTTGCAACAAGAAAGCACC
A3	GGAATTCTCGACATCAGCGATCATTCTTTGCAATTCCATGTTAGAATCCAATTCATCACCGATTCTTCTCAAACATTCAGACAATTTTTTGGTAGAAGCATCTTGTG
B1	GGAATTCGCTGATGTCGATACCGATTCTCCAAGAGAAGTCTTCTTCAGAGTCGCTGCTGATATGTTCGCTGATGGTAACTTCAACTG
B2	AATTCTGGGACTTTGGTACACAAAGCTTTCAAGACCAATTTAGAAGCGAAGTAGAACAAAGCGACGACTCTACCCCAGTTGAAGTTACCA
B3	CCACCTTGATCTTGGATCCAGACCAACAATCTTTCTCTCAAGAAATCCAAGGTCCAACCCATGATGGTTCTGATCAATTCTGGGACTTTG
C1	ATTGTTGGTCTGGATCCAAGATCAAGGTGGTTGGGAAGGTTTGTTGTCTTACTTCGGTACCCCAACCTGGCAAACCGTCA
C2	TCCCCCGGGGGA**TTA**ACCCATTTTTTTCCAGATGGTCAAAGAAGCGGTCAAGACACCAGCGACGAAGATGGTGACGGTTTGCCAGGTTGGG

Start and stop codons are in bold; restriction sites used for cloning are underlined.

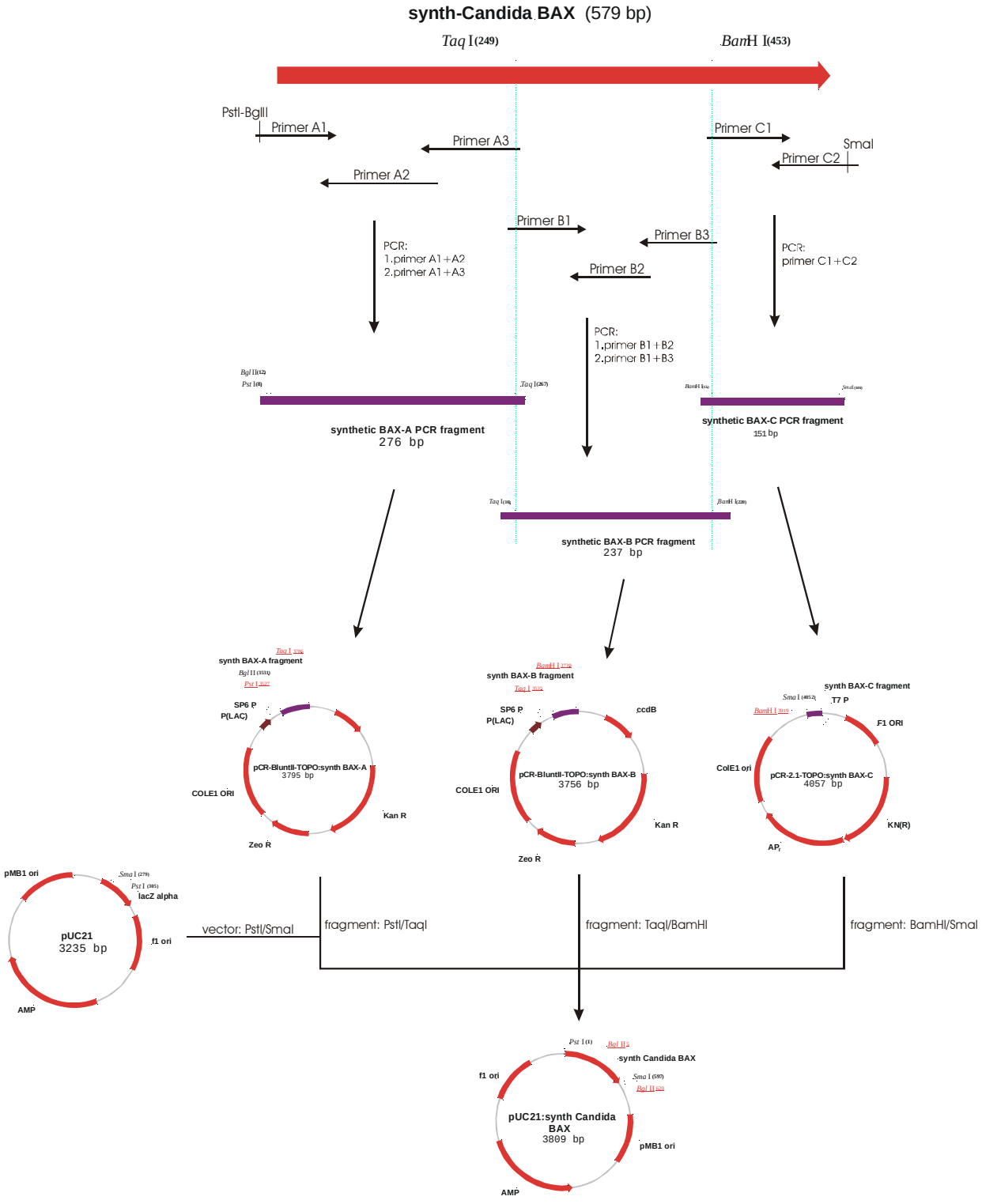

Figure 1. Outline for the synthesis of the synthetic *BAX* gene, using *C. albicans* optimal codons

Fragments A and B were synthesized in two steps. In the first PCR, primers X1 and X2 (X represents A or B, respectively) were used together. The resulting fragment served as a template for a second PCR performed with primers X1 and X3. Fragment C was synthesized in an independent PCR using primers C1 and C2. Fragments A and B were cloned into the pCR-BluntII-TOPO vector (Stratagene Cloning Systems, La Jolla, CA), whereas fragment C was cloned into the pCR2.1-TOPO vector (Stratagene Cloning Systems, La Jolla, CA) (Fig. 1). All three fragments were sequence verified.

Subsequently, fragment A was digested with *Pst*I and *Taq*I, fragment B with *Taq*I and *Bam*HI, and fragment C with *Bam*HI and *Sma*I. The three products were cloned in a quadruple ligation into pUC21 digested with *Pst*I and *Sma*I, to produce plasmid pUC21:synthCandidaBAX. The sequence of the synthetic *BAX* gene is shown in Figure 2.

```
     M   D   G   S   G   E   Q   L   G   S   G   G   P   T   S
  1  ATG GAT GGT TCT GGT GAA CAA TTG GGT TCT GGT GGT CCA ACC TCT

     S   E   Q   I   M   K   T   G   A   F   L   L   Q   G   F
 46  TCT GAA CAA ATC ATG AAA ACC GGT GCT TTC TTG TTG CAA GGT TTC

     I   Q   D   R   A   G   R   M   A   G   E   T   P   E   L
 91  ATC CAA GAT AGA GCT GGT AGA ATG GCT GGT GAA ACC CCA GAA TTG

     T   L   E   Q   P   P   Q   D   A   S   T   K   K   L   S
136  ACC TTG GAA CAA CCA CCA CAA GAT GCT TCT ACC AAA AAA TTG TCT

     E   C   L   R   R   I   G   D   E   L   D   S   N   M   E
181  GAA TGT TTG AGA AGA ATC GGT GAT GAA TTG GAT TCT AAC ATG GAA

     L   Q   R   M   I   A   D   V   D   T   D   S   P   R   E
226  TTG CAA AGA ATG ATC GCT GAT GTC GAT ACC GAT TCT CCA AGA GAA

     V   F   F   R   V   A   A   D   M   F   A   D   G   N   F
271  GTC TTC TTC AGA GTC GCT GCT GAT ATG TTC GCT GAT GGT AAC TTC

     N   W   G   R   V   V   A   L   F   Y   F   A   S   K   L
316  AAC TGG GGT AGA GTC GTC GCT TTG TTC TAC TTC GCT TCT AAA TTG

     V   L   K   A   L   C   T   K   V   P   E   L   I   R   T
361  GTC TTG AAA GCT TTG TGT ACC AAA GTC CCA GAA TTG ATC AGA ACC

     I   M   G   W   T   L   D   F   L   R   E   R   L   L   V
406  ATC ATG GGT TGG ACC TTG GAT TTC TTG AGA GAA AGA TTG TTG GTC

     W   I   Q   D   Q   G   G   W   E   G   L   L   S   Y   F
451  TGG ATC CAA GAT CAA GGT GGT TGG GAA GGT TTG TTG TCT TAC TTC

     G   T   P   T   W   Q   T   V   T   I   F   V   A   G   V
496  GGT ACC CCA ACC TGG CAA ACC GTC ACC ATC TTC GTC GCT GGT GTC

     L   T   A   S   L   T   I   W   K   K   M   G   *
541  TTG ACC GCT TCT TTG ACC ATC TGG AAA AAA ATG GGT TAA
```

Figure 2. DNA and protein sequences of the synthetic *Candida Bax* gene.

Construction of synthetic BAX- and GFP-synthetic BAX expression plasmids

A *Pst*I-*Sma*I fragment containing the ORF of the synthetic *BAX* gene was cloned into the *Pst*I-*Stu*I digested vector pGAL1ACT1LUC (W. Martinet, unpublished), to produce the expression construct pGAL1P:synthCaBAX (Fig. 3A). To facilitate recognition of the AUG codon during formation of initiation complexes, a purine base (A) was introduced at position –3 from the AUG codon (Kozak, 1981) using the Quick change site directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA).

The yeast enhanced *GFP* gene (yE*GFP*) (Cormack *et al.*, 1997) was amplified by PCR using upstream primer 5'-AACTGCAG**ATG**TCTAAAGGTGAAGAATTATTC-3' and downstream

primer 5'-GGAAGATCTTCCTTTGTACAATTCATCCATACC-3'. The upstream primer introduced a *Pst*I site (underlined), while the downstream primer contained a *Bgl*II linker (underlined) for fusion with the synthetic *BAX* gene. After cloning of the yE*GFP* gene into the pCR2.1-TOPO vector, the gene was sequence verified.

The yE*GFP*-synth *Candida BAX* fusion was created by cloning a *Pst*I-*Bgl*II yE*GFP* fragment together with a *Bgl*II-*Sma*I synthetic *Candida BAX* fragment into the *Pst*I-*Stu*I digested expression vector pGAL1ACT1LUC. The pGAL1P:yEGFP-synthCaBAX fusion construct (Fig. 3B) that was obtained was sequence verified.

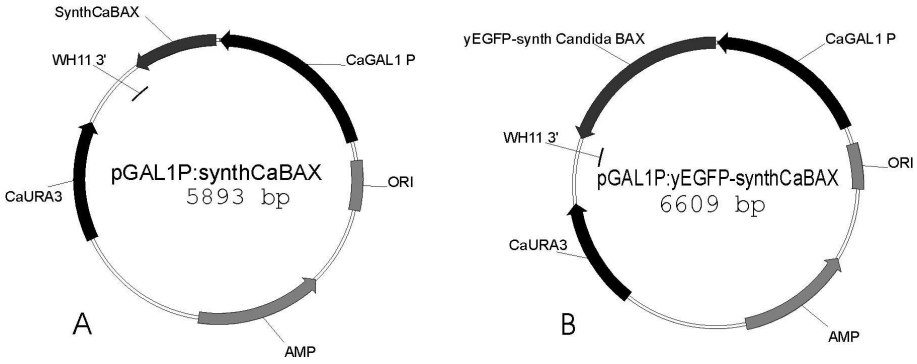

Figure 3. Representation of the expression constructs for the synthetic *CaBAX* gene (A) and the yEGFP-synth *CaBAX* fusion (B).

Creation of the synthetic BAX expression strains

C. albicans was transformed with the expression plasmids using a modified version (M. Logghe, unpublished) of the spheroplasting protocol (Herreros *et al.*, 1992). The plasmids were linearized with *Bpu*1102I to allow directed integration into the genome at the *GAL1* promoter site. Correct integration was confirmed by Southern blotting. Genomic DNA was prepared from different transformants using the Nucleon® extraction and purification kit (Amersham Pharmacia Biotech, Rainham, UK), and then digested with *Xba*I. The BAX probe was prepared by PCR using pGAL1P:synthCaBAX as template, the sense primer 5'-ATGGATGGTTCTGGTGAAC-3' and the anti-sense primer 5'-TTAACCCATTTTTTTCCAGATG-3'. Standard PCR conditions were used. For detection of yE*GFP*, a probe was synthesized by PCR using sense primer 5'-AGAGATCTCGAGGGATCC-3' and anti-sense primer 5'-GCATTATTTGTACAATTCATCC-3'. Southern blot hybridization and detection were performed using the AlkPhos DIRECT labelling and detection system (Amersham Pharmacia Biotech, Rainham, UK) following the instructions of the manufacturer.

Western blot analysis

Cells cultured overnight in SD-ura medium to late log phase were harvested by centrifugation, washed twice with water and resuspended in SG-ura to induce Bax expression. After 15 h of

induction, crude cell extracts were prepared as described (Sambrook, 1989). Bax protein was detected using a polyclonal rabbit antibody against mouse and rat Bax protein (Pharmingen, San Diego, CA). Due to contamination of this antibody with antibodies against yeast cell wall mannan, a very high background signal was obtained. This background was effectively reduced by pre-adsorbing the antibody with 0.5 mg/ml purified yeast mannan (Rossanese *et al*., 1999). Gfp protein was detected with a mouse monoclonal anti-Gfp antibody (Molecular Probes, Eugene, OR). For the time kinetic experiment, cells were grown and induced as described. Samples of the cultures were taken at several time points (0, 1, 3, 6, 9, 12 and 24 h) after resuspension in SG-ura medium. Crude extracts were prepared as described above, and samples of 30 µg protein were analyzed by Western Blot. The Bax protein was detected using a polyclonal rabbit anti-Bax antibody (Abcam, Cambridge, UK). The Gfp-Bax fusion protein was detected with a mouse monoclonal anti-Gfp antibody (Molecular Probes, Eugene, OR).

Growth curves

Yeast cells were grown for 24 h in SD-ura medium (supplemented with uridine for the wild type). Cells were harvested, washed twice with water and resuspended to an OD_{600} of 0.0125 in 400 µl of fresh SD-ura or SG-ura medium. Growth was monitored in Honey-Well plates using the Bioscreen C system (Labsystems, Helsinki, Finland).

Viability tests

Cells were pre-grown in minimal dextrose medium to an OD_{600} of 1.0. The cells were then washed twice with water and switched to minimal medium containing galactose as carbon source. At the time points indicated, equal amounts of cells (based on measurement of OD_{600}) were spread on minimal dextrose plates and incubated at 30°C for counting of viable cells.

Localization studies

The intracellular localization of Gfp-Bax was investigated in a double-staining experiment using MitoTracker Red CM-H_2XROS (Molecular Probes, Eugene, OR) for mitochondrial staining, and Hoechst 33258 (Molecular Probes, Eugene, OR) for nuclear staining. Gfp-Bax transformants were grown overnight in SD-ura, washed twice with water, resuspended in SG-ura to induce expression of the fusion protein, and incubated for 17 hours. After induction, $2x10^7$ cells were washed with 1 mM potassium phosphate buffer pH 7.4 (PPB) and incubated with 150 nM MitoTracker Red in 1 mM PPB for 10 min at 37°C. Cells were washed with 1 mM PPB and incubated for 5 min with Hoechst 33258 in 1 mM PPB. Microscope slides were prepared and examined using fluorescence microscopy.

Results

Conditional expression of the synthetic BAX gene in Candida albicans

A cDNA encoding the full-length mouse Bax protein was placed under control of the *C. albicans GAL1* promoter, allowing conditional expression when cells are grown in media containing galactose. Initial experiments were performed using the wild type mouse *BAX* gene. Expression of this gene did not result in any detectable phenotype; no difference in growth compared to the wild type was observed when cells were grown in media containing galactose (data not shown). This could have been due to the different codon usage by *C. albicans* and related species (Santos *et al.*, 1990; Ohama *et al.*, 1993; Santos *et al.*, 1993). These species use a tRNA with a CAG anticodon to decode CUG as serine, whereas most organisms use CAG to decode CUG as leucine. The mouse *BAX* gene contains 9 in frame CUG codons, making it most unlikely that a functional Bax protein could be expressed in *C. albicans*. Further analysis of the codons used in the mouse *BAX* gene revealed non-optimal codon usage for *Candida* (http://alces.med.umn.edu/candida/codons.html). To ensure a high level of expression of the *BAX* gene, a codon-adapted synthetic version of the gene was created by a PCR-based strategy using the primers in Table 1. The synthetic *BAX* gene was fused to the yE*GFP* to allow FACS screening for transformants expressing high levels of yEGfp-synthCaBax.

The resultant plasmids, pGAL1P:synthCaBAX and pGAL1:GFP-synthCaBAX, were transformed into the *C. albicans* CAI-4 strain. Transformants were selected on uridine-free minimal medium. About 25 transformants of each expression construct were plated on minimal dextrose medium (non-inducing conditions) and on minimal galactose medium (inducing conditions), and incubated at 30ºC for two days, All transformants grew on the medium containing dextrose, but most of them failed to grow when galactose was used as a sole carbon source (Fig. 4A). Southern blot analysis of the transformants revealed that, in transformants that were unable to grow on galactose as carbon source, a copy of the synth*CaBAX* gene had been integrated into the endogenous copy of the *GAL1* promoter. The transformants that were able to grow on galactose as carbon source did not have the correct integration (data not shown). To study differences in growth, the transformants were grown overnight in medium containing synthetic dextrose, washed with water, and transferred to fresh medium containing galactose as carbon source. The wild type strain grew well on medium containing galactose, but the transformants expressing Bax did not (Fig. 4B and C).

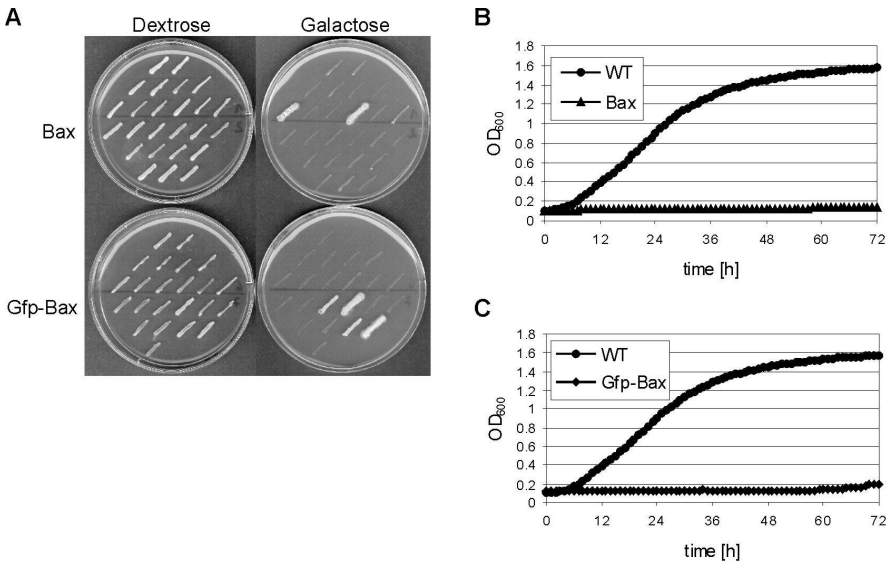

Figure 4. (A) Growth of the *Candida albicans* transformants: individual transformants of pGAL1P:synthCaBAX and pGAL1P:GFP-synthCaBAX were plated onto medium containing either 2 % dextrose or 2% galactose as sole carbon source. Growth was evaluated 4 days later. (B) Growth kinetics of GAL1P:synthCaBAX and (C) GAL1P:GFP-synthCaBAX on minimal medium containing galactose.

Western blot analysis of the synthCaBAX transformants showed accumulation of the Bax protein (15 h Bax induction, Fig. 5A). A very faint band of approximately the same molecular weight is visible in the wild type strain (*C. albicans* CAI-4). This band could be a yeast cell wall mannan. Detected as a result of the contamination of the anti-Bax antibody with antibodies against yeast cell wall mannan (as mentioned in Materials and Methods). When immunoblotting was performed with the strains expressing Gfp-Bax, a fusion protein with the expected molecular mass of 48 kDa was detected under inducing conditions (galactose as carbon source). In addition to the fusion protein, a protein band of about 27 kDa was also detected, which corresponds to the molecular mass of the Gfp protein alone. Addition of a Gfp-expressing strain as a positive control to the Western blot confirmed these results; the Gfp protein was detected at the same molecular mass as the unexpected band in the strains expressing Gfp-synthCaBax (Fig. 5B). This is most probably due to proteolytic cleavage of the fusion protein. Analysis of Gfp-fluorescence using FACS showed a high signal in the transformants expressing the fusion protein (data not shown).

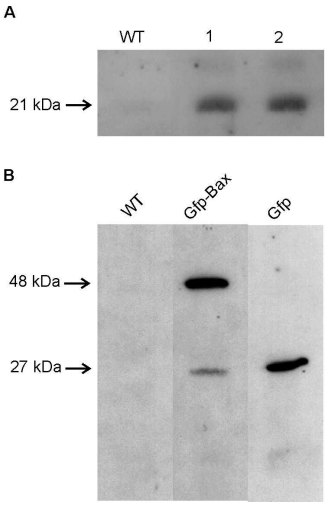

Figure 5. (A) Immunoblot analysis, using anti-Bax antibody, of two independent transformants (1 and 2) of GAL1P:synthCaBAX after 15 h Bax induction in minimal medium containing galactose. The arrow at 21 kDa indicates the position of the Bax protein. WT: *C. albicans* CAI-4. (B) Immunoblot analysis, using anti-Gfp antibody, of the GAL1P:GFP-synthCaBAX strain (Gfp-Bax) on minimal medium containing galactose. The band appearing at 48 kDa represents the Gfp-Bax fusion protein, whereas the band at 27 kDa represents the Gfp protein alone. WT: *C. albicans* CAI-4. Gfp : Gfp-expressing strain.

When cell viability was analyzed, different results were obtained for the synthCaBAX strain and the GFP-synthCaBAX strain (Fig. 6). In the strain expressing the Gfp-synthCaBax fusion protein, almost all the cells died during the first 3 hours of incubation in medium containing galactose as sole carbon source. This contrasts with the strain expressing synthCaBax, in which quite a rapid decrease in the number of colony forming units occurred during the first 6 hours of incubation on medium containing galactose, after which the rate of death slowed down significantly.

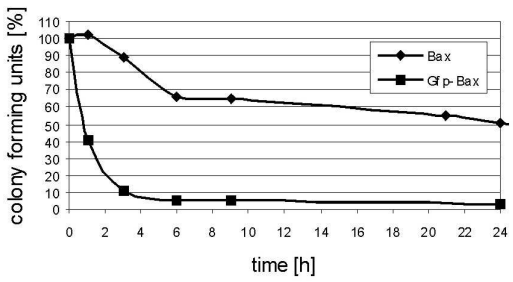

Figure 6. Viability test for synthCaBAX and GFP-synthCaBAX transformed cells: Cells were pre-grown in minimal dextrose medium and then switched to fresh minimal medium containing galactose. At the indicated time points, equal amounts of cells were spread on minimal dextrose plates. The colonies that appeared represent the viable fraction of cells of the total pool.

The Bax trigger in the cells expressing synthCaBax may not have been strong enough to kill all the cells, or else accumulation of Bax to a required threshold value was slow enough to allow the cells to activate some type of defence mechanism, possibly based on proteolytic degradation of the Bax protein. Notably, the situation was different for the fusion protein. Gfp itself is a very stable protein (data not shown), and it might increase the stability of a protein to which it is

fused, in this case Bax. That would translate into a rapid accumulation of Bax (fused to Gfp) and a stronger death trigger; the cells die faster, even before their defence mechanism could be activated.

Stability of the Bax and Gfp-Bax proteins

To evaluate the hypothesis that Gfp stabilizes Bax in the Gfp-Bax fusion protein, Western blots were made with crude extracts of cells expressing Gfp-Bax or Bax at several time points after induction of expression. Both proteins were expressed within 1 h of induction. In the case of Bax, protein accumulation was maximal after 6 hours, after which it decreased spectacularly, and became almost undetectable after 24 h (Fig. 7A). These results concur with the results of the viability test (Fig. 6), where the rapid decrease in cell viability during the first 6 h of induction was followed by a much less pronounced decrease, leading to survival of approximately 50% of the cells after 24 h of induction. It appears that the cells defend themselves against Bax-induced cell death by the proteolytic degradation of Bax. This contrasts with the expression of the Gfp-Bax fusion protein, which accumulated over time. Even after 24 h of induction, little proteolytic degradation was observed. The very stable Gfp protein stabilizes Bax, and leads to a stronger death trigger that results in survival of only a few percent of the cells after 24 h of induction (Fig. 6).

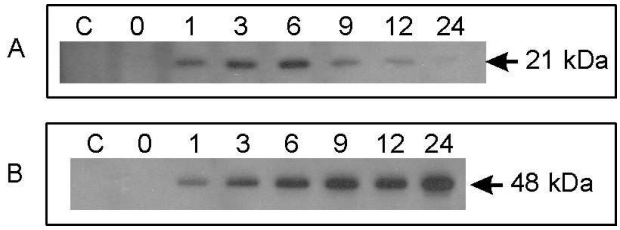

Figure 7. Immunoblot analysis to check the expression levels of Bax (A) and Gfp-Bax (B). Cells were grown in dextrose medium and then transferred to galactose medium to start induction. Cells were induced for of 24 h and samples were taken at several time points. C: control.

Gfp-Bax localizes to the mitochondria

The intracellular location of the Gfp-Bax fusion protein was examined in a double-staining localization experiment. The mitochondria were stained with MitoTracker Red, and the nucleus with Hoechst 33258, and localization was visualized with fluorescence microscopy. In cells expressing Gfp, this protein is diffusely distributed throughout the entire cell, and the mitochondria show a typical distribution pattern over the whole cell as well (Fig. 8A). In cells expressing Gfp-Bax, however (Fig. 8B and C), the fusion protein is located in a single cluster, and the mitochondria are also clustered instead of dispersed. About 85% of the Gfp-Bax

expressing cells that were stained, showed this mitochondrial relocalization. When the pictures are merged, it becomes clear that the Gfp-Bax fusion protein coincides with the mitochondria, which aggregate next to the nucleus. This indicates that in *C. albicans* Gfp-Bax localizes to the mitochondria, which translocate to a single cluster in the perinuclear region.

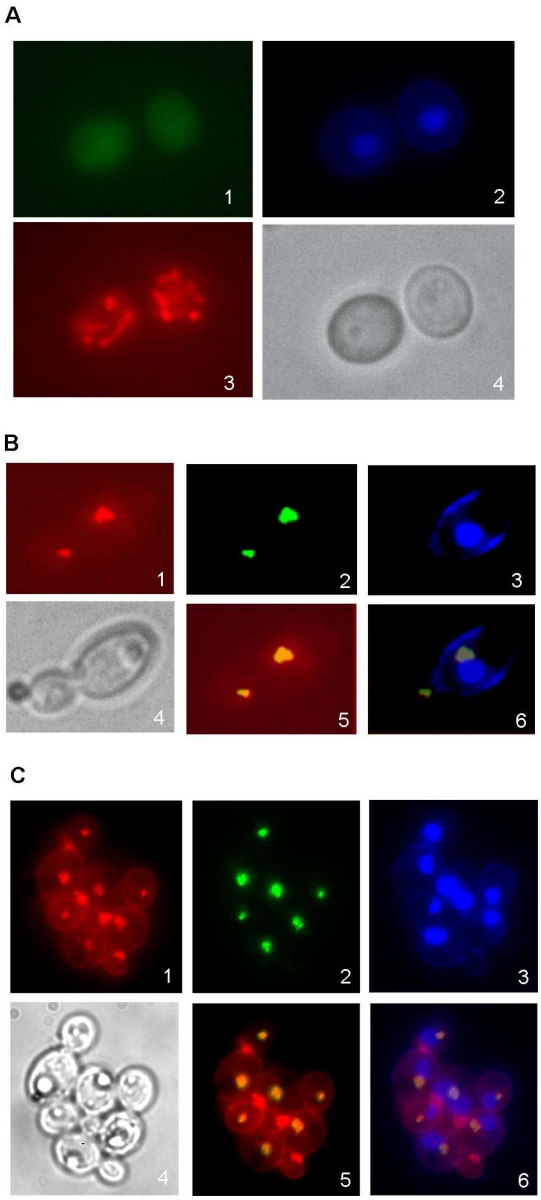

Figure 8. (A) Gfp and mitochondrial localization in cells expressing Gfp. 1: Gfp localization, 2: nuclear staining, 3: mitochondrial staining, 4: phase contrast image. (B and C) Perinuclear clustering of the mitochondria upon Gfp-Bax expression. 1: mitochondrial staining; 2: Gfp-Bax localization; 3: nuclear staining; 4: phase contrast image; 5: overlay of 1 and 2; 6: overlay of 1, 2 and 3.

Discussion

A number of groups have demonstrated that Bax induces cell death in several yeast species: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and the methylotrophic yeast *Pichia pastoris* (Sato *et al.*, 1994; Jurgensmeier *et al.*, 1997; Madeo *et al.*, 1997; Martinet *et al.*, 1999). In this study, we investigated for the first time the effect of Bax expression in the pathogenic yeast *C. albicans*. Identification of genes involved in Bax-induced cell death in *C. albicans* could eventually lead to the development of new antifungal therapeutics.

When expressing heterologous proteins in *C. albicans* it is important to compare the codons used in the gene of interest with the codon usage of *Candida spp.*, which differs in some aspects from regular codon usage. The most important peculiarity is the use of a tRNA with a CAG anticodon to decode CUG as serine, rather than leucine as in most organisms. Consequently, expression of the mouse *BAX* gene, which contains 9 CTG codons, is highly unlikely to produce a functional Bax protein in *C. albicans*. Other codons in the mouse *BAX* also seemed to be suboptimal for producing a functional protein. Therefore, a fully synthetic gene was constructed, and its expression yielded a functional Bax protein. This synthetic gene was also fused to yE*GFP*, and the expressed fusion protein also possessed Bax activity. Transformants were unable to grow on medium with galactose as sole carbon source, indicating that the expression of Bax and Gfp-Bax inhibits growth. Western blot and FACS analyses showed accumulation of Bax and Gfp-Bax in the transformants. Western blot analysis of the strain expressing Gfp-Bax revealed two bands: an expected band of the fusion protein at 48 kDa and an additional band at 27 kDa of Gfp alone, indicating partial proteolytic cleavage of the fusion protein at or near the junction between the two proteins. This phenomenon was also observed with other Gfp fusion constructs containing the same *Bgl*II linker (unpublished results).

Bax expression not only inhibited growth of *C. albicans*, but also induced its death. Bax induced cell death drastically increased when Bax was fused to Gfp. We have noticed that Gfp is a very stable protein, and its stability might have stabilized the Gfp-Bax fusion protein and led to its accumulation in the cells. However, Bax alone was degraded rapidly. This may explain the difference in cytotoxicity between Gfp-Bax and Bax, the latter of which might have been unable to reach a necessary threshold in all the cells. It is also conceivable that by the time Bax reached a level sufficient to trigger cell death, the cells had already activated a defence mechanism, possibly proteolytic degradation of Bax, thereby limiting cell death. In contrast, the very stable nature of the fusion protein allows Bax (fused to Gfp) to rapidly reach the threshold to trigger cell death in almost all the cells before they had time to activate a defence mechanism; this results in a dramatic induction of a cell death program.

It is well documented that upon induction of apoptosis in mammalian cells, Bax localizes to the mitochondria (Wolter *et al.*, 1997). The same is true for yeast cells (Priault *et al.*, 1999). Removal of the C-terminal transmembrane domain prevented targeting of Bax to the

mitochondria in yeast and abolished the cytotoxic effect of Bax in yeast cells (Zha *et al.*, 1996). We demonstrate that in *C. albicans* a Gfp-Bax fusion protein localizes to the mitochondria, which translocate from a dispersed distribution in the cell to a single cluster in the perinuclear region. There are no previous reports on perinuclear mitochondrial clustering in yeast upon Bax expression. However, several studies with mammalian cells illustrate mitochondrial clustering, for example upon treatment with cell death inducers such as TNFα (De Vos *et al.*, 1998; Esposti *et al.*, 1999; Maianski *et al.*, 2003), TRAIL (Thomas *et al.*, 2000), ceramide (Maianski *et al.*, 2003) and Concanavalin A (Suen *et al.*, 2000). Mitochondrial clustering also occurs upon infection of HeLa cells with herpes simplex virus (Murata *et al.*, 2000), and by overexpression of some proteins, for example BID (a pro-apoptotic member of the Bcl-2 family) (Li *et al.*, 1998) and cRel (a transcription factor of the Rel/NF-κB family) (Bernard *et al.*, 2001). Translocation of mitochondria to the perinuclear region is an early feature of the cell death response, and seems to be independent of the mechanism (necrosis or apoptosis). Even though translocation of mitochondria requires intact microtubules, the driving force is not the microtubules *per se*, but the loss of activity of the mitochondria-associated molecular motor kinesin. Inhibition of mitochondrial translocation to a perinuclear region by destruction of the microtubules or by immunoinhibition of kinesin, markedly delayed cell death (De Vos *et al.*, 1998; Murata *et al.*, 2000; Thomas *et al.*, 2000). Thomas and colleagues (2000) suggested that perinuclear clustering facilitates cell death by concentrating molecules that induce cell death close to their targets in the nucleus. Bernard *et al.* (2001), however, proposed that mitochondria cluster because they are damaged by the H_2O_2 they overproduce. Damaged mitochondria would subsequently be degraded by autophagocytosis. We show that Gfp-Bax expressed in *C. albicans* localizes to the mitochondria, where it probably causes mitochondrial damage and leads to their translocation to a perinuclear region. In *S. cerevisiae*, Bax expression leads to the formation of ROS that oxidatively damage the mitochondria (Madeo *et al.*, 1999), but mitochondrial clustering was not reported.

In this study we report for the first time the effects of Bax expression on the pathogenic yeast *C. albicans*. Expression of Bax leads to inhibition of growth and cell death. A Gfp-Bax fusion protein localizes to the mitochondria and induces translocation of the mitochondria from a disperse distribution in the cell to a single cluster in the perinuclear region. This is the first time that mitochondrial clustering was observed to accompany cell death in yeast. Further work is needed to elucidate the molecular mechanism underlying this cell death process and the accompanying mitochondrial perinuclear aggregation. The identification of functions involved in this cell death process might eventually lead to the development of new antifungal therapeutics.

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2 Histatine 5-geïnduceerde celdood in *Candida albicans*

2.1 Inleiding

Door de conditionele expressie van Bax hebben we een celdoodproces opgang kunnen brengen in *C. albicans*. Een nadeel van dit celdoodproces is dat het op gang gebracht wordt door de intracellulaire expressie van een heteroloog gen. Een meer fysiologisch relevant celdoodproces werd gevonden in het gebruik van een peptide dat bij de mens in het speeksel voorkomt, waar het onder meer instaat voor het voorkomen van infecties in de mondholte. Dit natuurlijk voorkomend peptide is histatine 5.

Histatine 5 behoort tot een familie van kleine, kationische, histidine-rijke peptiden met een moleculair gewicht van 3 tot 4 kDa die voorkomen in het speeksel van hogere primaten (MacKay *et al.*, 1984b). De histatine peptiden worden constitutief geproduceerd en gesecreteerd door de onderkaakspeekselklier, sublinguale speekselklier en oorspeekselklier (vanderSpek *et al.*, 1989; Ahmad *et al.*, 2004). Deze histidine-rijke peptiden werden voor het eerst beschreven in de vroege jaren ’70 als peptiden die de glycolytische activiteit van microorganismen zouden verhogen (Holbrook & Molan, 1973). Later werd hun bactericidale (MacKay *et al.*, 1984a) en fungicidale activiteit beschreven (Pollock *et al.*, 1984). De histatines maken deel uit van het aangeboren immuunsysteem en spelen een belangrijke rol in het behoud van de orale gezondheid door het verhinderen van infecties in de mondholte. De histatine familie bestaat uit verschillende leden (Castagnola *et al.*, 2004), waarvan histatine 1, 3 and 5 de belangrijkste zijn. Histatine 5 is een peptide van 24 aminozuren lang met 7 histidine residuen (DSHAKRHHGYKRKFHEKHHSHRGY) (Oppenheim *et al.*, 1988) en heeft als theoretisch moleculair gewicht 3034,51 Da. Karakterisatie van de secundaire structuur toonde aan dat in waterige oplossing histatine 5 een *random coil* structuur aanneemt en overgaat naar een structuur met een α-helix in niet-waterige oplossingen (Raj *et al.*, 1998). Van al de histatines beschikt histatine 5 over de sterkste antimicrobiële activiteit en het grootste deel van het onderzoek naar histatines is gebeurd op histatine 5.

In het begin van het onderzoek naar dit peptide werd zuiver peptide geïsoleerd uit speeksel (Oppenheim *et al.*, 1988; Xu *et al.*, 1991), later kon het geproduceerd worden door heterologe expressie in bacteriën (Tsai *et al.*, 1996), de laatste jaren is men echter overgegaan naar chemische synthese (Helmerhorst *et al.*, 1997). Deze recombinante en chemisch gesynthetiseerde peptiden bleken dezelfde secundaire structuur en antimicrobiële activiteit te hebben als het natuurlijke histatine 5.

In dit onderzoek werd geopteerd om het peptide chemisch te synthetiseren. De synthese van histatine 5 en de controle van het syntheseproduct werd uitgevoerd op de Vakgroep Biochemie van Prof. Vandekerckhove.

2.2 Materiaal en methoden

2.2.1 Stammen en media

Candida albicans CAI-4 (*ura3::imm434/ura3::imm434)* werd ons vriendelijk beschikbaar gesteld door Dr. W. Fonzi (Georgetown University, Washington, DC) (Fonzi & Irwin, 1993). *S. cerevisiae* INV-Sc1 (MAT*a leu2-3,-112, trp1-289, ura3-52, his?1*) werd aangekocht bij Invitrogen (San Diego, CA). Synthetisch dextrose medium bevat 0.67% (w/v) yeast nitrogen base zonder aminozuren, 2% (w/v) glucose en 0.079% (w/v) *complete supplement mixture* (BIO 101 Inc., Carlsbad, CA). YPD medium bevat 1% (w/v) yeast extract, 2% (w/v) pepton en 2% (w/v) glucose. Om platen te maken werd aan het medium 2% (w/v) agar toegevoegd. Om groei te verzekeren van *C. albians* CAI-4, werd uridine aan het medium toegevoegd tot een concentratie van 20 µg/ml.

2.2.2 Synthese en zuivering van histatine 5

Histatine 5 (DSHAKRHHGYKRKFHEKHHSHRGY) werd gesynthetiseerd en opgezuiverd door de Vakgroep Biochemie van Prof. Vandekerckhove (Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent). Synthese gebeurde zoals eerder beschreven (Vancompernolle *et al.*, 1992) met een model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) op Wang resin (p-benzyloxybenzyl alcohol resin– Advanced Chemtech), door gebruik te maken van de Fmoc-chemie procedure en volgens de instucties van de producent. Het gesynthetiseerde peptide werd gezuiverd door middel van preparatieve HPLC (TFA-acetonitril systeem), en de sequentie werd gecontroleerd aan de hand van MALDI-TOF massaspectrometrie (reflex III-Daltonik GmbH, Bremen, Germany) (Gevaert *et al.*, 1998). Het peptide werd gelyofiliseerd en bewaard bij -18°C.

2.2.3 Circulair Dichroïsme

Circulair dichroïsme spectra werden opgenomen bij kamertemperatuur in het verre UV-gebied (184-260 nm) met een JASCO J-170 spectropolarimeter. Histatine 5 werd opgelost in een concentratie van 100 µM in 10 mM Na-fosfaat buffer pH 7,4 of in 100% trifluoroethanol (TFE). Voor elk staal werd het gemiddelde van 9 spectra genomen. De spectra werden gecorrigeerd voor achtergrondwaarden.

2.2.4 Overlevingstesten

Overlevingstesten werden uitgevoerd zoals eerder beschreven (Raj *et al.*, 1990) met de nodige aanpassingen aan het protocol. *C. albicans* of *S. cerevisiae* cellen werden geïnoculeerd in SD medium en gegroeid tot vroege exponentiële fase bij 30°C en 250 tpm. Cellen werden tweemaal gewassen met 10 mM Na-fosfaat buffer pH 7,4 en geresuspendeerd in dezelfde buffer met een

celdensiteit van ongeveer 5 x 10^7 cellen/ml. 150 µl van deze celsuspentie werd gemengd met 150 µl van een dubbele concentratie histatine 5 opgelost in 10 mM Na-fosfaat buffer pH 7,4. Dit mengsel werd al schuddend geïncubeerd bij 30°C (finale celdensiteit is 2.5 x 10^7 cellen/ml). Op verschillende tijdsintervallen werden stalen genomen, verdund en uitgeplaat op YPD. Deze platen werden gedurende 48 uur geïncubeerd bij 30°C. Kolonies werden manueel geteld en het percentage overleving werd berekend (kolonies tijdpunt t_x/kolonies tijdpunt t_0) x 100.

2.3 Resultaten

2.3.1 Synthese en zuivering van histatine 5

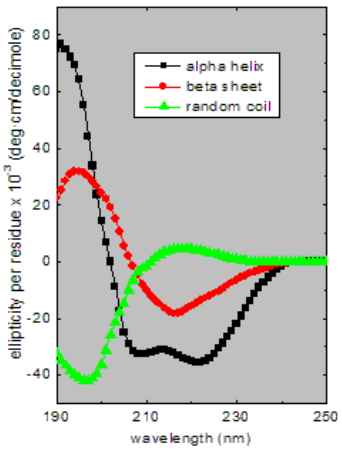
Synthese van het histatine 5 peptide werd uitgevoerd door de mensen van het laboratorium van Prof. Vandekerckhove (Vakgroep Biochemie, Universiteit Gent). Het histatine 5 peptide is 24 aminozuren lang, bevat 7 histidine residuën (29,2 %) en heeft als sequentie NH_2-DSHAKRHHGYKRKFHEKHHSHRGY-OH. Het theoretisch moleculair gewicht is 3034,51 Da. De synthese gebeurde zoals beschreven in de materiaal en methoden sectie van dit hoofdstuk. Na zuivering werd de sequentie gecontroleerd door MALDI-TOF massaspectrometrie. Het moleculair gewicht en de sequentie bleken correct.

2.3.2 Circulair Dichroïsme

Bij de start van dit project werd er door verschillende groepen onderzoek gedaan naar de structuur van histatine 5 en de rol van de structuur voor zijn functie, aangezien men ervan uit ging dat de structuur van het peptide belangrijk zou zijn voor zijn werking. Histatine 5 bleek een *random coil* structuur aan te nemen in waterig milieu en in hydrofoob milieu een α-helicale structuur.

Om na te gaan of ook de voor ons gesynthetiseerde peptiden deze secundaire structuren vertonen, die van belang kunnen zijn voor zijn werking, hebben we CD-spectra opgenomen van dit peptide in waterig milieu (Na-fosfaat buffer) en in hydrofoob milieu (TFE).

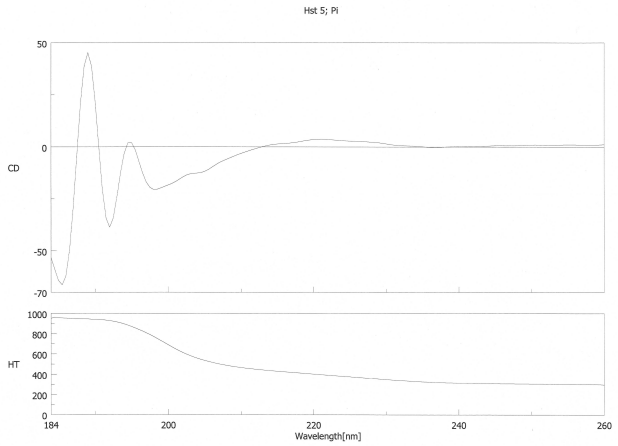
Figuur 1 toont enkele typische CD-spectra voor een *random coil*, α-helicale en β-*sheet* structuur. Hierbij is vooral het gebied tussen 200 nm en 235 nm belangrijk. Een peptide met een α-helicale structuur vertoont een spectrum met twee minima, één minimum bij ongeveer 207 nm en één minimum bij ongeveer 222 nm. Indien het peptide een β-*sheet* structuur aanneemt dan vertoont het CD-spectrum één minimum dat ongeveer gelegen is bij 215 nm. Een peptide dat een *random coil* structuur aanneemt, vertoont een zwak maximum bij ongeveer 217 nm.



Figuur 1: CD-spectra voor α-helix, β-*sheet* en *random coil* structuren.

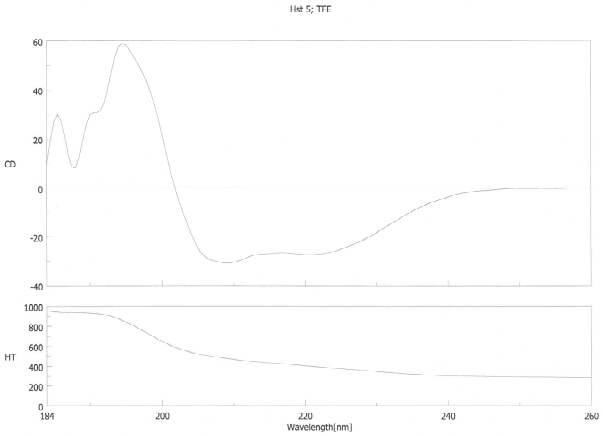
Het synthetisch histatine 5 werd opgelost in Na-fosfaat buffer of TFE in een concentratie van 100 µM en de CD-spectra van deze oplossingen werden opgenomen. De verkregen spectra vertonen een mooie grafiek vanaf ongeveer 200 nm. De oplossingen bevatten waarschijnlijk onzuiverheden die het beeld van het spectrum verstoren tussen 184 en 200 nm.

Uit de metingen blijkt dat het peptide in waterig milieu (buffer) een spectrum vertoont met een zeer zwak maximum rond 220 nm, wat erop duidt dat het synthetisch histatine 5 voornamelijk een *random coil* structuur aanneemt in 10 mM Na-fosfaat buffer (Fig. 2).



Figuur 2: CD-spectrum van 100 µM synthetisch histatine 5 opgelost in 10 mM Na-fosfaat buffer.

Indien het synthetisch histatine 5 opgelost is in TFE dan vertoont het spectrum twee minima, één rond 208 nm en één rond 222 nm (Fig.2). Dit is het typisch spectrum van een peptide met een α-helicale structuur.

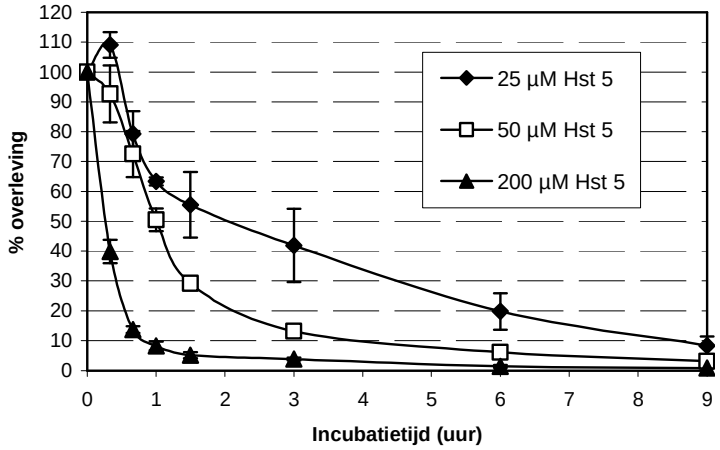


Figuur 3: CD-spectrum van 100 µM synthetisch histatine 5 opgelost in 100% TFE.

2.3.3 Overlevingstesten

We hebben nu een synthetisch peptide in handen waarvan de sequentie en de secundaire structuur overeenkomen met deze van het natuurlijk voorkomend histatine 5. De volgende stap is nagaan of dit synthetisch peptide een candidacidale activiteit vertoont die vergelijkbaar is met het natuurlijke histatine 5. Om dit te bereiken werd eerst een protocol uitgewerkt dat voor ons goed werkbaar is en waarmee relatief veel cellen behandeld kunnen worden.

De *C. albicans* CAI-4 stam werd gedurende enkele uren geïncubeerd met verschillende concentraties van het synthetisch histatine 5 volgens het protocol vermeld in de materiaal in methoden sectie van dit hoofdstuk. Het synthetische histatine 5 blijkt een sterke candidacidale activiteit te vertonen die concentratie afhankelijk is (Fig. 4). Na 90 minuten incubatie met 25 µM histatine 5 is er een overleving van 55,5%. Als de concentratie verhoogd wordt tot 50 µM dan overleven gedurende dezelfde tijdsspanne 29,2% van de cellen. Incubatie in de aanwezigheid 200 µM histatine 5 gedurende 90 minuten levert een overleving op van slechts 5,1 %. Deze activiteit is vergelijkbaar met deze vermeld in verschillende publicaties. Indien de cellen geïncubeerd worden in dezelfde omstandigheden maar in afwezigheid van peptide dan treedt er geen celdood op (data niet getoond).

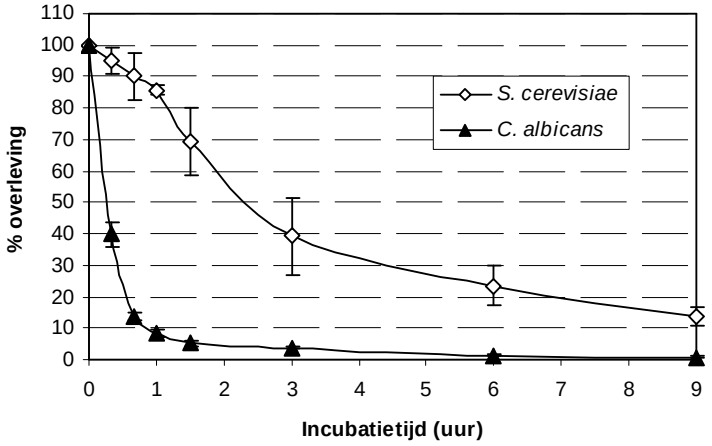


Figuur 4: Candidacidale activiteit van verschillende concentraties van het synthetisch histatine 5 tegen *C. albicans* CAI-4. De resultaten zijn een gemiddelde met standaard deviatie van 3 onafhankelijke experimenten.

De niet-pathogene gist *S. cerevisiae* wordt dikwijls gebruikt als modelorganisme in het onderzoek naar *C. albicans* vanwege zijn verwantschap met *C. albicans* en vanwege de vele moleculaire en genetische *tools* voorhanden voor het onderzoek naar *S. cerevisiae*.

Om na te gaan of bakkergist dezelfde gevoeligheid vertoont voor histatine 5, werd de *S. cerevisiae* INV-Sc1 stam geïncubeerd met 200 µM histatine 5 en werd de gevoeligheid voor histatine 5 vergeleken met deze van *C. albicans* (Fig. 5). Uit de resultaten blijkt dat *S. cerevisiae*

veel resistenter is voor histatine 5 vergeleken met *Candida*. Na 90 minuten incubatie met peptide overleven 69,4% van de *S. cerevisiae* cellen. Dit in tegenstelling met *C. albicans* waar onder dezelfde omstandigheden slechts 5,1% van de cellen overleven.



Figuur 5: Fungicidale activiteit van 200 µM histatine 5 tegen *S. cerevisiae* INV-Sc1 en *C. albicans* CAI-4. De resultaten zijn een gemiddelde met standaard deviatie van 3 onafhankelijke experimenten.

2.4 Besluit

In dit hoofdstuk beschrijven we de synthese en karakterisatie van het antifungale peptide histatine 5. Aan de hand van CD-spectra hebben we aangetoond dat het gesynthetiseerde peptide dezelfde secundaire structuur bezit als het natuurlijke peptide: *random coil* in waterig milieu en α-helicaal in hydrofoob milieu. Verder werd een protocol opgesteld om in *C. albicans* en *S. cerevisiae* een celdoodproces te initiëren, door incubatie van de cellen met histatine 5. *Candida* blijkt zeer gevoelig te zijn voor dit peptide en deze gevoeligheid is concentratieafhankelijk. Vergeleken met *Candida* is *Saccharomyces* veel resistenter voor histatine 5. Dit verschil in gevoeligheid voor histatine 5 werd reeds eerder beschreven (Edgerton *et al.*, 1998) een verklaring werd er echter niet gegeven. Een mogelijke verklaring voor dit verschil in gevoeligheid zal in het volgende hoofdstuk gegeven worden.

Alhoewel we reeds een bruikbaar celdood initiërend systeem opgezet hadden in *C. albicans* door de intracellulaire expressie van Bax, hebben toch verder gezocht naar een ander systeem. Dit hebben we gevonden door de extracellulaire incubatie van de cellen met het peptide, histatine 5. Twee belangrijke voordelen zijn aan dit nieuwe systeem verbonden. 1) Het is een natuurlijk voorkomend celdoodproces. Histatine 5 komt voor in het speeksel bij hogere primaten waar het ondermeer instaat voor het verhinderen van bacteriële en fungale infecties in de mondholte. De conditionele heterologe expressie van Bax in *Candida*, daarentegen, zorgt voor een artificieel celdoodproces, aangezien Bax niet voorkomt in gistcellen. 2) Histatine 5 wordt extracellulair aan de cellen toegevoegd. Dit heeft als voordeel dat de binding van het peptide aan de cel (al dan niet aan een receptor), internalisatie van het peptide en eventuele signalisatie naar latere evenementen in het celdoodproces ook onderzocht kunnen worden.

2.5 Referenties

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3 Rol van oxidatieve fosforylatie in het histatine 5-geïnduceerde celdoodproces in *Saccharomyces cerevisiae*

In het vorige hoofdstuk van deze resultatensectie hebben we aangetoond dat we een efficiënte afdoding verkrijgen van *C. albicans* cellen wanneer deze in contact gebracht worden met histatine 5. Daarnaast bleek dat *S. cerevisiae* veel resistenter is voor histatine 5-geïnduceerde celdood. Ook in de literatuur werd reeds melding gemaakt van de hogere resistentie van *S. cerevisiae* voor histatine 5-geïnduceerde celdood (Edgerton *et al.* 1998). In dit hoofdstuk pogen we te verklaren hoe dit verschil in gevoeligheid van deze twee gisten voor histatine 5 te verklaren is.

Ondanks de grote gelijkenissen tussen *C. albicans* en *S. cerevisiae* en het feit dat bakkersgist in veel onderzoeksprojecten gebruikt wordt als modelorganisme voor het onderzoek naar *C. albicans*, is er een groot verschil tussen beide gisten. *S. cerevisiae* is een *Crabtree*-positieve gist; wanneer bakkersgist gegroeid wordt op een fermenteerbare koolstofbron onder aerobe condities, dan worden de suikers voornamelijk gefermenteerd tot ethanol. *C. albicans*, daarentegen, is een *Crabtree*-negatieve gist; indien *C. albicans* gegroeid wordt onder aerobe condities zullen de cellen respiratief groeien, zelfs indien de koostofbron fermenteerbaar is. Voor *C. albicans* werd reeds beschreven dat oxidatieve fosforylatie noodzakelijk is om histatine 5-geïnduceerde celdood te ondergaan (Helmerhorst *et al.* 1999). Is dit ook het geval voor andere gisten? Bijvoorbeeld *S. cerevisiae*? Kan het verschil in gevoeligheid te wijten zijn aan en verschil in activiteit van de mitochondriën? Op deze vragen geven we in dit hoofdstuk een antwoord. De resultaten van dit onderzoek werden gepubliceerd in *Biotechnology Letters.*

De gevoeligheid van *S. cerevisiae* voor histatine 5 werd bepaald na een voorgroei van de cellen in fermenteerbare en niet-fermenteerbare koolstofbronnen en in de aan- of afwezigheid van carbonyl cyanide m-chlorofenylhydrazon (CCCP), een ontkoppelaar van de oxidatieve fosforylatie. *S. cerevisiae* bleek resistenter voor histatine 5 indien de cellen gegroeid werden in een fermenteerbare koostofbron dan wanneer groei gebeurde in een niet-fermenteerbare koolstofbron. Dit wijst op een belangrijke rol voor de oxidatieve fosforylatie in het histatine 5-geïnduceerde celdoodproces. Oxidatieve fosforylatie is noodzakelijk voor histatine 5-geïnduceerde celdood in *Candida albicans*, dit bleek echter niet het geval te zijn voor *S. cerevisiae*. Incubatie van CCCP-behandelde *S. cerevisiae* cellen met histatine 5 resulteerde nog altijd in celdood. Deze resultaten suggereren dat histatine 5-geïnduceerde celdood in *S. cerevisiae* verschilt van histatine 5-geïnduceerde celdood in *C. albicans*

Artikel

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Role of oxidative phosphorylation in histatin 5-induced cell death in *Saccharomyces cerevisiae*

Kris De Smet, Rieka Reekmans & Roland Contreras*

Unit of Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, B-9052 Ghent, Belgium

*Author for correspondence (Fax: ++32 9 3313502 ; E-mail: Roland.Contreras@dmbr.UGent.be)

Key words: *Candida albicans,* cell death, histatin 5**,** oxidative phosphorylation, *Saccharomyces cerevisiae*

Abstract

The succeptibility of *Saccharomyces cerevisiae* to the antimicrobial peptide, histatin 5, was tested after pre-growth in fermentable and non-fermentable carbon sources and in the absence or presence of the uncoupler of oxidative phosphorylation, carbonyl cyanide m-chlorophenylhydrazone (CCCP). *S. cerevisiae* was more resistant to histatin 5 when grown on a fermentable carbon source compared to growth on a non-fermentable carbon source, indicating an important role for oxidative phosphorylation in histatin 5-induced cell death. Oxidative phosphorylation is a prerequisite for histatin 5-induced cell death in *Candida albicans* but this is not the case in *S. cerevisiae*. Incubation of CCCP-treated *S. cerevisiae* cells with histatin 5 still resulted in cell death. These results suggest that histatin 5-induced cell death in *S. cerevisiae* differs from that in *C. albicans*.

Introduction

Most higher organisms produce peptides with potent antifungal and antibacterial activity as part of their innate immune system (Boman, 1998). In humans, histatins, a family of anti-microbial peptides, are produced by the submandibular and parotid glands and secreted in the saliva (Oppenheim *et al.*, 1988). These peptides play an important role in maintaining oral health by limiting infections in the oral cavity. Histatins are small, cationic, histidine-rich peptides of 3-4 kDa with potent anti-microbial activity (Pollock *et al.*, 1984). The histatin family consists of 12 members, of which histatins 3 and 5 (32 and 24 amino acids, respectively) are the most important. Histatin 5 has the strongest antimicrobial activity and most of the research on histatins has focused on this peptide. It has potent antifungal activity against the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Tsai & Bobek, 1997; Helmerhorst *et al.*, 1999).

The mechanism of action of histatin 5 in *C. albicans* is a multistep process. Histatin 5 binds to a cell envelope protein (Li *et al.*, 2003) and, after internalisation, it is targeted to the mitochondria (Helmerhorst *et al.*, 1999). Once internalized, histatin 5 induces the efflux of ATP and K^+ ions from the cell into the extracellular medium. The extracellular ATP is thought to interact with a purinergic receptor, which in its activated form induces cell death (Koshlukova *et al.*, 1999; Koshlukova *et al.*, 2000). The activity of histatin 5 on *C. albicans* requires coupled mitochondria performing oxidative phosphorylation (Helmerhorst *et al.*, 1999). *C. albicans* mutants deficient in respiration due to mutations in mitochondrial DNA, so-called petite mutants, are much more resistant to histatin 5-induced cell death (Gyurko *et al.*, 2000). Uncoupling of oxidative phosphorylation by carbonyl cyanide m-chlorophenylhydrazone (CCCP) or by azide results in protection against histatin 5-induced cell death (Koshlukova *et al.*, 1999). Thus, histatin 5 seems to target the respiratory apparatus. Increased levels of reactive oxygen species are detected in *C. albicans* cells treated with histatin 5 (Helmerhorst *et al.*, 2001). However, these increased levels of reactive oxygen species do not seem to play a direct role in fungicidal activity (Wunder *et al.*, 2004).

Because histatin 5 is active against several pathogenic yeasts, we have determined whether oxidative phosphorylation is important for histatin 5-induced cell death in other yeast species besides *C. albicans*. In this study the non-pathogenic yeast, *S. cerevisiae,* was used. *S. cerevisiae* is often used as a model organism for *C. albicans* because of the molecular and genetic tools available and because of its close relationship with *C. albicans*.

Materials and methods

Strains and media

Candida albicans CAI-4 (*ura3::imm434/ura3::imm434)* was kindly provided by Dr. W. Fonzi (Georgetown University, Washington, DC) (Fonzi & Irwin, 1993). *S. cerevisiae* INV-Sc1 (MAT*α leu2-3,-112, trp1-289, ura3-52, hisΔ1*) was purchased from Invitrogen (San Diego, CA). *S. cerevisiae* BY4742 (MAT*α ura3Δ, leu2, his3Δ, MET15, lys2Δ*) was purchased from Euroscarf (Frankfurt, Germany). Synthetic dextrose medium contains 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose and 0.079% (w/v) complete supplement mixture (BIO 101 Inc., Carlsbad, CA). Synthetic glycerol medium contains 0.67% (w/v) yeast nitrogen base without amino acids, 3% (v/v) glycerol and 0.079% (w/v) complete supplement mixture (BIO 101 Inc., Carlsbad, CA). YPD medium contains 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose. To ensure growth of *C. albians* CAI-4, uridine was added to 20 µg/ml. CCCP was purchased from Sigma-Aldrich (Irvine, UK).

Synthesis and purification of peptide

Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) was synthesized as previously described (Vancompernolle *et al.*, 1992) with a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) on Wang resin (p-benzyloxybenzyl alcohol resin– Advanced Chemtech), using the Fmoc chemistry procedure and following the manufacturer's instructions. The peptide was purified using preparative HPLC (TFA-acetonitrile system), and the sequence was verified by MALDI-TOF-MS (reflex III-Daltonik GmbH, Bremen, Germany) (Gevaert *et al.*, 1998).

Survival assay

Survival assays were performed as described previously (Raj *et al.*, 1990) with some modifications. Briefly, *C. albicans* or *S. cerevisiae* cells were inoculated into synthetic dextrose or synthetic glycerol medium and grown with vigorous shaking at 30 °C until early log phase. Cells were washed twice with 10 mM sodium phosphate buffer pH 7.4, and resuspended in 10 mM sodium phosphate buffer pH 7.4 at a cell density of approxi 5 x 10^7 cells/ml. 150 µl of cell suspension was mixed with 150 µl of a two-fold concentration of histatin 5 in 10 mM sodium phosphate buffer pH 7.4, and incubated at 30 °C with shaking (2.5 x 10^7 cells/ml). At intervals culture samples were diluted, plated on YPD agar and incubated for 48 h at 30 °C. Colonies were counted using a colony counter (LemnaTec, Würselen, Germany). Control cultures were incubated in the absence of histatin 5. The percent cell survival was calculated with the equation (colonies time point t_x/colonies time point t_0) x 100. For assaying survival of cells treated with CCCP, 5 x 10^7 cells/ml were incubated with 500 µM CCCP in 10 mM sodium phosphate buffer, pH 7.4, for 2 h at 30 °C. Cells were then incubated in the presence of 200 µM histatin 5. As the

CCCP stock solution was made with ethanol, final ethanol concentration in the cultures was 0.5% (v/v). Control cultures were first incubated for 2 h in 10 mM sodium phosphate buffer pH 7.4 with 0.5% ethanol (v/v), and then treated with 200 μM histatin 5. Experiments were done at least in triplicate.

Results and discussion

Saccharomyces cerevisiae is killed by histatin 5 but is less susceptible to it than *C. albicans* (Edgerton *et al.*, 1998). We tested the susceptibility of two *S. cerevisiae* strains (INV-Sc1 and BY4742) for histatin 5-induced cell death (Fig. 1). Cells were grown in a fermentable carbon source (glucose) and *C. albicans* CAI-4 was used as a control. Both strains of *S. cerevisiae* were susceptible to histatin 5 but much less so than *C. albicans*. With 200 µM histatin 5, survival after 1.5 h was about 5% for *C. albicans* and approximately 70% for both *S. cerevisiae* strains. Survival curves were comparable when 200 µM histatin 5 was used for *S. cerevisiae* and 25 µM histatin 5 for *C. albicans*. Thus, to achieve the same killing rate in both yeasts, the histatin 5 dose that was used with *S. cerevisiae* had to be eight-fold higher than that used with *C. albicans*. Unlike Edgerton *et al.* (Edgerton *et al.*, 1998), we could not observe a sub-population of *S. cerevisiae* cells that were unresponsive to histatin 5. This could be due to differences in experimental set-up or to the use of different *S. cerevisiae* strains.

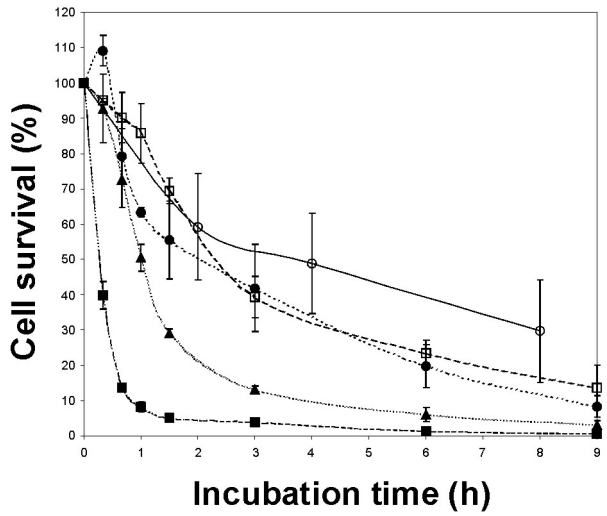

Figure 1: Killing activity, after glucose pre-growth of cultures, of histatin 5 to *C. albicans* with 25 µM (●), 50 µM (▲) or 200 µM (■) peptide and *S. cerevisiae* INV-Sc1 (□) and BY4742 (○) with 200 µM peptide. 7.5 x 10^6 cells were treated with histatin 5. At intervals culture samples were diluted, plated on YPD agar and incubated for 48 h at 30 °C. The data shown are means and standard deviations of at least three experiments.

S. cerevisiae is often used as a model organism for *C. albicans,* because of the availability of molecular and genetic tools, and because of its close relationship to *C. albicans*. However, there is an important difference between the two yeasts. *S. cerevisiae* is a Crabtree-positive yeast; when grown on a fermentable carbon source under aerobic conditions, the sugar is largely

fermented to ethanol rather than respired. *C. albicans*, however, is a Crabtree-negative yeast; when grown under aerobic conditions the cells respire, even when the carbon source is fermentable. This difference can explain the observed difference in susceptibility to histatin 5. For this peptide to exert its fungicidal action, active mitochondria must be present. Grown aerobically on glucose, a fermentable carbon source, *S. cerevisiae* cells will largely perform fermentation. Consequently, the mitochondria are not active and do not perform oxidative phosphorylation, which results in lower susceptibility to histatin 5. By contrast, *C. albicans* cells do perform oxidative phosphorylation on a fermentable carbon source under aerobic conditions, which makes them more sensitive to histatin 5-induced cell death.

To verify this hypothesis we performed a survival assay after pre-growth on a non-fermentable carbon source (glycerol). When grown on glycerol the cells have to perform oxidative phosphorylation to generate energy. After glycerol pre-growth the sensitivity of *S. cerevisiae* cells increased spectacularly (Fig. 2a). After 1 h incubation with 200 µM histatin 5, cell survival was only 5% when pre-growth was on glycerol, compared to 70% when pre-growth was on glucose. For *C. albicans* cells (Fig. 2b) there was also an increase in sensitivity, manifested in a reduction in survival from 51% after glucose pre-growth to 21% after glycerol pre-growth (1 h incubation with 50 µM histatin 5). This increase in susceptibility might have been due to the limiting O_2 concentration in the cultures, which in the case of glucose cultures might have been so low that respirative growth alone could not provide enough energy, and part of the energy was derived from fermentation. In glycerol, however, no fermentation is possible, and all the energy has to come from oxidative phosphorylation. Thus, the two growth conditions result in different oxidative phosphorylation levels, which in turn lead to different sensitivities to histatin 5.

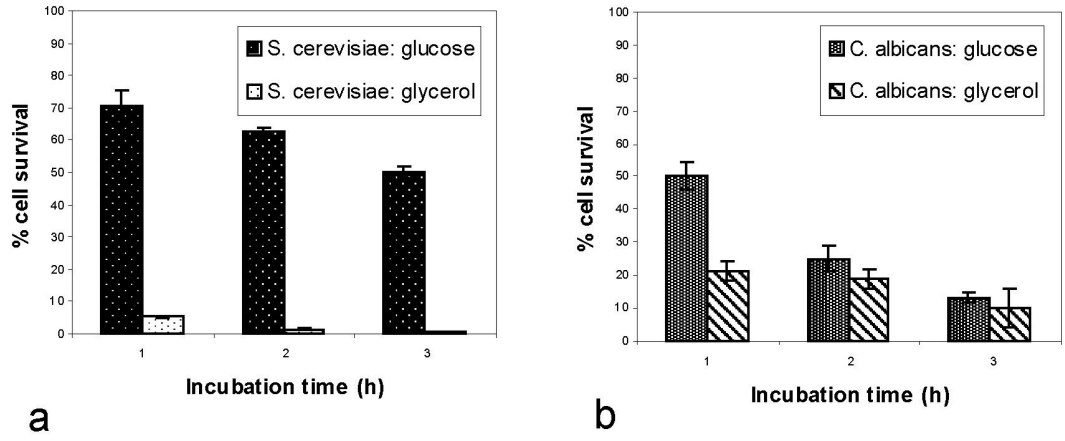

Figure 2: Killing activity, after glucose or glycerol pre-growth of cultures, of histatin 5 to *S. cerevisiae* BY4742 with 200 µM peptide (a) and *C. albicans* CAI-4 with 50 µM peptide (b). 7.5 x 10^6 cells were treated with histatin 5. At intervals culture samples were diluted, plated on YPD agar and incubated for 48 h at 30 °C. The data shown are means and standard deviations of at least three experiments.

In *C. albicans* oxidative phosphorylation is a prerequisite for histatin 5-induced cell death (Helmerhorst *et al.*, 1999). However, 50% of the *S. cereviae* cells also die after a 3-h histatin 5 incubation when grown fermentatively (Fig. 2a). To confirm these results, a survival assay was performed using cells treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a potent uncoupler of oxidative phosphorylation. *S. cerevisiae* and *C. albicans* cells were first incubated for 2 h with 500 µM CCCP and then treated with 200 µM histatin 5 (Fig. 3). Uncoupling of oxidative phosphorylation prevented histatin 5-induced cell death in *C. albicans*, as about 95% of the cells survived a 4-h incubation with the peptide. When CCCP was omitted from the culture, approximately 15% survived a 4 h incubation with histatin 5. In contrast, uncoupling of oxidative phosphorylation had no effect on the survival of *S. cerevisiae* cells after incubation with histatin 5, indicating that it did not alter susceptibility to histatin 5-induced cell death. Non-treated cells died as fast as treated cells (average cell survival was 25% and 30%, respectively, after 4 h histatin 5 incubation). To check the toxicity of CCCP, an experiment was performed in which histatin 5 was omitted. At the CCCP concentration used, no loss of cell viability was observed (data not shown).

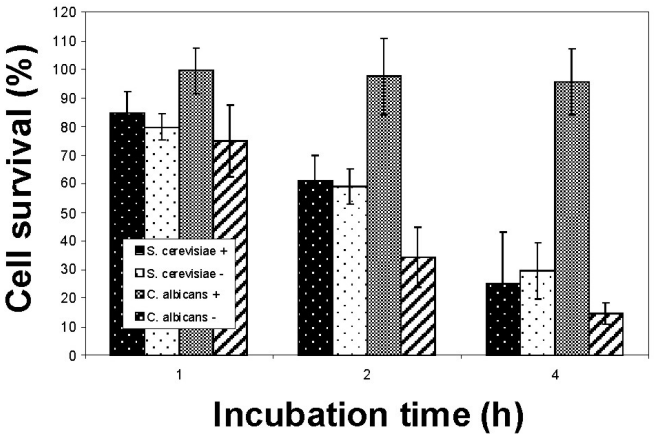

Figure 3: Killing activity of histatin 5 to *C. albicans* CAI-4 and *S. cerevisiae* BY4742 cells treated with CCCP. After glucose pre-growth the cells were incubated for 2 h in the presence (+) or absence (-) of 500 µM CCCP and were then treated with 200 µM histatin 5. 7.5 x 10^6 cells were treated with histatin 5. At intervals culture samples were diluted, plated on YPD agar and incubated for 48 h at 30 °C. The data shown are means and standard deviations of at least three experiments.

Conclusion: Previous studies indicate that *S. cerevisiae* is much more resistant to histatin 5 compared to *C. albicans*. In these studies cells were grown on a fermentable carbon source. Our results reveal that, when grown on a non-fermentable carbon source, *S. cerevisiae* cells are much more susceptible to histatin 5. Although oxidative phosphorylation is important for histatin 5-induced cell death in *S. cerevisiae*, it is not a prerequisite. In *C. albicans* oxidative phosphorylation is absolutely necessary for histatin 5-induced cell death. This difference points to an additional mechanism for histatin 5-induced cell death in *S. cerevisiae* that is not present in *C. albicans*. Therefore *S. cerevisiae* should not be used as a model organism for studying cell death induced by histatin 5.

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4 Identificatie van genen betrokken bij het histatine 5-geïnduceerde celdoodproces in *Candida albicans*

In hoofdstuk 2 hebben we aangetoond dat we een celdoodproces kunnen initiëren in *C. albicans* door de cellen te incuberen met histatine 5. Door de identificatie van genen die betrokken zijn bij het histatine 5-geïnduceerde celdoodproces kunnen we het werkingsmechanisme van histatine 5 mogelijk verder ophelderen. Bovendien kunnen de genen die betrokken zijn bij dit celdoodproces gebruikt worden als doelwit voor de ontwikkeling van nieuwe antifungale stoffen. Met behulp van een overexpressiebibliotheek en microarray analyse hebben we enkele van deze genen kunnen identificeren. Zestig genen vertoonden een verandering in expressieniveau met tenminste een factor 2, na incubatie van de cellen met histatine 5. Overexpressie analyse van een selectie van deze genen toonde aan dat zij een invloed hebben op het celdoodproces. Vier van deze genen zijn betrokken bij mitochondriale respiratie of oxidatieve stress (*POR1*, *CAT2*, *PGA3* and *TRX1*), twee hebben een functie in *nutrient sensing* (*GPR1* and *SNF31*), één heeft een onbekende functie (*IPF2973*), en een ander is betrokken bij stress (*DDR48*).

De resultaten van dit werk zijn neergeschreven in manuscriptvorm en dit manuscript vormt de basis voor een artikel dat ingediend is ter publicatie bij het tijdschrift *Eukaryotic Cell*.

Manuscript

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Identification of genes involved in histatin 5-induced cell death in *Candida albicans*

Kris De Smet[a,1], Petra Tiels[a,2], Joël Vandekerkhove[b], Patrick Van Dijck[c] and Roland Contreras[a,*]

[a] Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and Flanders Interuniversity Institute for Biotechnology (VIB), Technologiepark 927, B-9052 Ghent, Belgium.

[b] Department of Medical Protein Research, Ghent University-VIB, , Albert Baertsoenkaai 3, B-9000 Ghent, Belgium. Phone ++32 9 264 93 17. Fax ++32 9 264 94 90. E-mail: joel.vandekerckhove@ugent.be

[c] Laboratory of Molecular Cell Biology, Katholieke Universiteit Leuven and Department of Molecular Microbiology-VIB, Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium. Phone: ++32 16 32 15 12. Fax: ++32 16 32 19 79. E-mail: Patrick.vandijck@bio.kuleuven.ac.be

* Corresponding author. Mailing address: Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, Ghent University and VIB, Technologiepark 927, B-9052 Ghent, Belgium. Phone: ++32 9 331 3631. Fax: ++32 9 331 3502. E-mail: Roland.Contreras@dmbr.UGent.be

[1] Current address: Flen Pharma NV, Research Department, Drie Eikenstraat 661, B-2650 Edegem, Belgium. Phone: ++32 3 825 70 63. Fax : ++32 3 226 46 58. E-mail: kris.desmet@flenpharma.com

[2] Current address: Department of Virology, Parasitology and Immunology, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Phone: ++32 9 264 73 84. Fax: ++32 9 264 74 96. E-mail: Petra.Tiels@UGent.be

Abstract

Histatin 5 is an antimicrobial peptide with potent candidacidal activity present in the saliva of humans. It forms part of the innate immune system and plays an important role in maintaining oral health. Histatin 5 seems to target the respiratory apparatus of *Candida albicans*, resulting in inhibition of respiration, formation of radical oxygen species (ROS), and finally cell death. Further, potassium channels and release of intracellular ATP seem to play a role in histatin 5-induced death of *C. albicans*. Identification of functions involved in histatin 5-induced cell death can help to resolve the mechanism of action of histatin 5. In order to develop novel antifungal compounds, functions involved in this cell death process can be used as targets. By screening an overexpression library and profiling transcription, we identified functions involved in histatin 5-induced cell death in *C. albicans.* Sixty genes differentially regulated at least two-fold were identified. Overexpression analysis of some of these genes demonstrated their influence on cell death. Four of them are related to mitochondrial respiration or oxidative stress (*POR1*, *CAT2*, *PGA3* and *TRX1*), two are involved in nutrient sensing (*GPR1* and *SNF31*), one has an unknown function (*IPF2973*), and one is involved in stress response (*DDR48*).

Introduction

Most higher organisms produce peptides with potent antifungal and antibacterial activity as part of their innate immune system. These peptide antibiotics differ widely in amino acid composition, length and secondary structure. However, they all play essential roles in nonspecific host defenses by preventing or limiting infections. Most peptides exert their antifungal or antibacterial function by interacting with and destabilizing the microbial membrane (Bals, 2000). All these peptides have a broad range of biological properties. Besides their antimicrobial activity, they can influence inflammation, proliferation, wound healing, release of cytokines, homeostasis, chemotaxis and the preservation of the balance between proteases and protease inhibitors (Boman, 1998; De Lucca & Walsh, 1999; Bals, 2000; Selitrennikoff, 2001).

In humans, histatins, a family of antimicrobial peptides, are produced by the submandibular and parotid glands and are secreted in the saliva (Oppenheim *et al.*, 1986; Oppenheim *et al.*, 1988). They form part of the innate immune system and play an important role in maintaining oral health. Histatins are small, cationic, histidine-rich peptides of 3-4 kDa. They adhere to and inhibit the formation of hydroxyapatite (Richardson *et al.*, 1993), enhance the glycolytic activity of salivary flora (Holbrook & Molan, 1975), and have potent antimicrobial activity (Pollock *et al.*, 1984). The histatin family consists of several members, of which histatin 3 and histatin 5 (32 and 24 amino acids, respectively) are the most important. Histatin 5, which has the strongest antimicrobial activity, has received most of the attention. It has potent antifungal activity against the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* (Tsai & Bobek, 1997a; Tsai & Bobek, 1997b; Helmerhorst *et al.*, 1999).

The mechanism of action of histatin 5 in *C. albicans* is being rapidly unraveled. Histatin 5 binds to the cell envelope protein, Ssa1/2 (Edgerton *et al.*, 1998; Edgerton *et al.*, 2000; Li *et al.*, 2003), and following internalization it is targeted to the mitochondria (Helmerhorst *et al.*, 1999). Once internalized, histatin 5 induces the efflux of ATP and potassium ions from the cell. The histatin 5-induced release of ATP occurs while the cells are still metabolically active and before cell lysis occurs. The extracellular ATP is thought to interact with a purinergic receptor, which upon activation induces cell death (Koshlukova *et al.*, 1999; Koshlukova *et al.*, 2000). Histatin 5 induces G1 cell cycle arrest and reduction in cell volume (Baev *et al.*, 2002). The effects of histatin 5 on *C. albicans* require active mitochondria and respiring cells performing oxidative phosphorylation (Helmerhorst *et al.*, 1999). Petite cells of *C. albicans*, which are deficient in respiration due to mutations in mitochondrial DNA, are much more resistant to histatin 5-induced cell death (Gyurko *et al.*, 2000). Further, uncoupling or blocking of cellular respiration by carbonyl cyanide m-chlorophenylhydrazone (CCCP) or by azide protects against histatin 5-induced cell death (Koshlukova *et al.*, 1999; De Smet *et al.*, 2004). Thus, histatin 5 seems to

target the respiratory apparatus of the cell, inhibiting respiration and causing formation of oxygen radicals (ROS). Cell death ensues due to oxidation of biologically important molecules and subsequent loss of cell integrity (Helmerhorst *et al.*, 2001). However, recent papers state that ROS do not play a role in the candidacidal activity of histatin 5 (Veerman *et al.*, 2004; Wunder *et al.*, 2004); the formation of ROS may be secondary to the effects of histatin 5 on processes influencing cellular metabolism or ion homeostasis.

Furthermore, potassium channels seem to play a role in histatin 5-induced death of *C. albicans*. The K^+ channel Tok1 plays a modulating role in histatin 5-induced cell death (Baev *et al.*, 2003), but it does not seem to be the primary site of histatin 5 action. Further work showed that decreased expression of Trk1 resulted in resistance to histatin 5-induced cell death. Trk1 seems to be essential for ATP loss, and is critical for the candidacidal activity of histatin 5 (Baev *et al.*, 2004).

The incidence of fungal infections is increasing, particularly in immunocompromised patients in whom the infection can be life threatening. *Candida albicans* remains the most prevalent cause of deep mycosis in humans. The number of effective, non-toxic antifungal compounds for human use is limited and resistance against the most frequently used antifungals (the azoles) is increasing (Boschman *et al.*, 1998). There is an urgent need for new fungicidal compounds that act on novel molecular targets.

The view that production of ROS is essential for histatin 5-induced cell death (Helmerhorst *et al.*, 2001), was recently questioned (Veerman *et al.*, 2004; Wunder *et al.*, 2004). The formation of ROS may be secondary to the effects of histatin 5 on cellular metabolism or ion homeostasis. Further, there is evidence that potassium channels play an important role in this cell death process (Baev *et al.*, 2003; Baev *et al.*, 2004). These recent papers emphasize that ATP release into the extracellular medium is critical for histatin 5-induced cell death, in contrast to the papers that stress the important role of mitochondria (Helmerhorst *et al.*, 1999; Gyurko *et al.*, 2000; Helmerhorst *et al.*, 2001; De Smet *et al.*, 2004). By screening an overexpression library and profiling transcription we sought to identify genes involved in the histatin 5-induced cell death process and to further clarify the molecular mechanisms involved in this cell death process of *C. albicans*. Genes involved in this cell death process can be used as targets for the development of new antifungal drugs.

Materials and methods

Strains and media

C. albicans CAI-4 (*ura3::imm434/ura3::imm434)* was kindly provided by Dr. W. Fonzi, Georgetown University (Washington, DC) (Fonzi & Irwin, 1993). *C. albicans GPR1* and *GPA2* deletion strains were described previously (Maidan *et al.*, 2005). Synthetic medium (SC) contains 0.67% (w/v) yeast nitrogen base without amino acids, 0.079% (w/v) complete supplement mixture minus uracil (BIO 101 Inc., Carlsbad, CA) and a carbon source: 2% (w/v) dextrose (SDC), 2% (w/v) galactose (SGC), 2% (w/v) sucrose (SSC), or 2% (w/v) mannose (SMC). YPD medium contains 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose. LYM medium contains 2% (w/v) dextrose, RPMI 1640 amino acid solution, RPMI 1640 vitamins solution (both from Sigma-Aldrich, Irvine, UK), 5.4 mM KCl, 5.6 mM Na_2PO_4, 0.5 mM magnesium sulfate and 1.0 mM sodium citrate. Each liter of medium is supplemented with 0.4 mg $ZnCl_2$, 2.0 mg $FeCl_2 \cdot 6H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $MnSO_4 \cdot H_2O$ and 0.1 mg $Na_2B_4O_7 \cdot 10H_2O$. To ensure growth of *C. albians* CAI-4, uridine was added to a final concentration of 20 µg/ml. *C. albicans* cells were maintained on YPD agar at 4°C. To prepare plates, media were solidified with 2% agar.

Synthesis and purification of peptide

Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) was synthesized as previously described (Vancompernolle *et al.*, 1992) with a model 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA) on Wang resin (p-Benzyloxybenzyl alcohol resin – Advanced Chemtech, KY) using the Fmoc chemistry procedure and following the manufacturer's instructions. The peptide was purified using preparative HPLC (TFA-acetonitrile system) and the sequence was verified by MALDI-TOF-MS (reflex III-Daltonik GmbH, Bremen, Germany) (Gevaert *et al.*, 1998).

Library screening

Construction of pRM2MAL2P2000SN - A blunted *Hin*P1I fragment from pMAL2PNiST-1 (J. Viaene, unpublished), containing the human *IFN*β gene flanked by a *Sfi*I and a *Not*I site, was cloned into the *Sma*I-*Sph*I opened and blunted vector pMAL2PmuBax (I. Eberhardt, unpublished), to produce pMAL2PSiNT-ATG. This vector was digested with *Eco*RI-*Hin*dIII to isolate the expression cassette (containing the Ca*MAL2* promotor, the h*IFN*β gene and the cytochrome c terminator of *S. cerevisiae*) and then blunted. This cassette was cloned into the *Bam*HI opened and blunted pRM2CaMET15 vector (Viaene *et al.*, 2000), to produce pRM2CaMET15MAL2PSiNT-1. In a blunt-blunt ligation the WH11 terminator of *C. albicans* from pCRW3 (Srikantha *et al.*, 2001) was cloned into pRM2CaMET15MAL2PSiNT-1 behind

the h*IFN*β gene, to produce pRM2MAL2PSiNWH11. Correct integration of the terminator into the vector was verified by sequence analysis. Finally, a *Sfi*I-*Not*I 2000 bp stuffer fragment was cloned into the *Sfi*I-*Not*I opened pRM2MAL2PSiNWH11, replacing the h*IFN*β gene. The resulting vector is pRM2MAL2P2000SN (Fig. 1a).

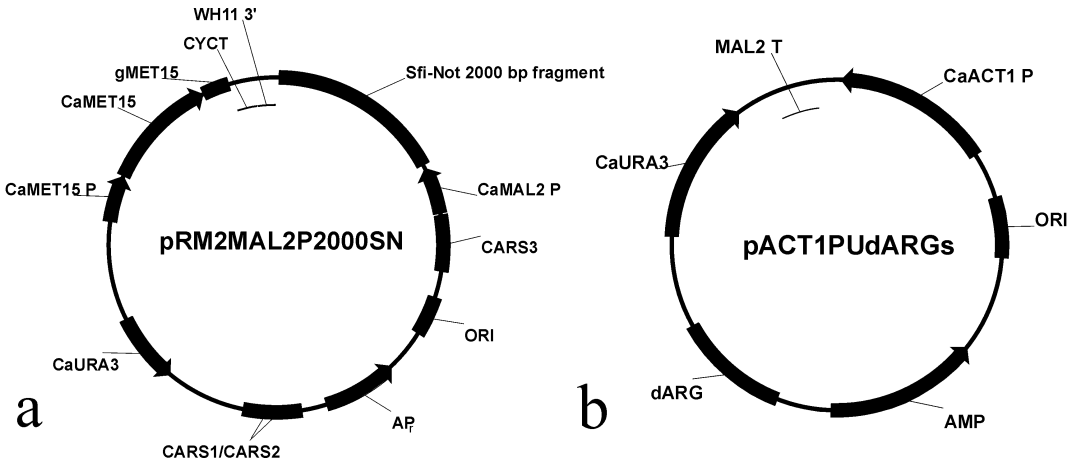

Figure 1: Key plasmids used in this study. (a) *C. albicans* replicative vector for the directional cloning of *C. albicans* cDNA behind the inducible *MAL2* promotor. (b) *C. albicans* integrative vector for the constitutive expression of histatin 5-responsive genes.

Construction of cDNA library – Total RNA was extracted from *C. albicans* CAI-4 grown in rich medium (YPD) using RNA-Pure™ Reagent (GenHunter Corporation, Nashville, TN). mRNA was prepared from total RNA using the mRNA purification kit of Amersham Pharmacia Biotech (Rainham, UK). First strand cDNA was synthesized with Superscript II Reverse Transcriptase (Life Technologies, Paisley, UK) and a *Not*I-Primer-Adaptor. After second strand synthesis, cDNA was polished with Klenow enzyme and purified over a Sephacryl S-400 spin column. Phosphorylated *Sfi*I adapters were ligated to the cDNA, followed by digestion with the *Not*I endonuclease. The *Sfi*I-*Not*I cDNA was finally purified over a Sepharose S-400 spin column. cDNA was ligated in the *Sfi*I-*Not*I opened pRM2MAL2P2000SN vector.

Histatin 5 treatment - The *C. albicans* cDNA library, cloned into the replicative pRM2MAL2P2000SN vector and transformed to *C. albicans* CAI-4, was inoculated in 50 ml SDC medium and grown overnight to early-log phase. The cells were washed with sterile H_2O, divided in two, and each part was resuspended in 50 ml of inducing medium (SSC or SMC). Both cultures were induced for 4 h. Cells were washed with 10 mM sodium phosphate buffer pH 7.4 and resuspended in this buffer at a cell density of $5x10^7$ cells/ml. 150 µl of this cell suspension and 150 µl of a 200 µM histatin 5 were combined and incubated for 2 h at 30°C and 250 rpm, and then 2 ml of inducing medium (SSC or SMC) were added. Surviving cells were left to recover overnight at 30°C, and were then inoculated in 50 ml inducing medium and

grown to early-log phase. They were again treated with histatin 5 as previously, left to recover overnight, and subjected to a third round of histatin 5 treatment. At time points 0 and 2 h of each treatment, 3 samples of ~625 cells were plated on SSC and on SMC agar, and incubated for 48 h at 30°C. Colonies were counted using a colony counter (LemnaTec, Würselen, Germany). The percentage of cell survival was calculated using the equation (colonies at time t_2 / colonies at time t_0) x 100. After the third round of histatin 5 treatment, all cells were plated on YPD and incubated for 48 h at 30°C. Single colonies were replica plated.

Individual survival assay - Survival assays were performed as described previously (Raj *et al.*, 1990), with some modifications. Briefly, *C. albicans* cells were inoculated into inducing medium (SSC or SMC) and grown with vigorous shaking at 30°C until early-log phase. Cells were washed twice with 10 mM sodium phosphate buffer pH 7.4 and resuspended in the same buffer at a density of approximately $5x10^7$ cells/ml. 150 µl of cell suspension and 150 µl of 200 µM histatin 5 (in 10 mM sodium phosphate buffer, pH 7.4) were combined and incubated at 30°C with shaking. At time points 0 and 2 h, cell suspensions were diluted and plated on YPD agar and incubated for 48 h at 30°C. Colonies were counted and the percentage of cell survival was calculated.

Identification of inserts - To recover plasmid DNA from yeast, cells were resuspended in 50 µl of STES (0.5 M NaCl, 0.2 M Tris-HCl, pH 7.6, 10 mM EDTA, 1% SDS). Glass beads were added and the cells were vortexed vigorously. After addition of 20 µl of H_2O and 60 µl of phenol/chloroform, the mixture was vortexed for 1 min and centrifuged at 14,000 g for 5 min. The DNA was precipitated with ethanol. The isolated DNA was retransformed into *E. coli* MC1061, plasmid DNA was isolated using NucleoBond PC-100 columns of Macherey-Nagel (Düren, Germany) and cDNA inserts were identified by sequencing.

Expression profiling using microarray technology

Histatin 5 treatment - *C. albicans* CAI-4 cells were grown in SDC + uridine at 30°C with vigorous shaking until early-log phase. Cells were washed twice with 10 mM sodium phosphate buffer pH 7.4 and resuspended in the same buffer at a cell density of $5x10^7$ cells/ml. Five ml of this cell suspension were added to 5 ml of 50 µM histatin 5 in 10 mM sodium phosphate buffer pH 7.4. Another 5 ml of this cell suspension were added to 5 ml of sodium phosphate buffer pH 7.4 (control culture). Both suspensions were agitated for 40 min at 30°C.

Total RNA extraction - After incubation, both cell suspensions (control and histatin 5-treated) were harvested and washed with ice-cold H_2O. The cells were combined with 1 ml of RNA-Pure™ Reagent (GenHunter Corporation, Nashville, TN) and 1 g of glass beads with a diameter of 0.5 mm, and broken by thorough mixing. One ml of chloroform was added and the suspension was centrifuged at 20,000 g for 10 min at 4°C. The RNA was precipitated for 10 min on ice with an equal volume of isopropanol, pelleted at 20,000 g for 10 min at 4°C, and washed

with 70% ice-cold ethanol. The RNA was resuspended in 40 µl RNAse-free H_2O. The purity and quality of the RNA was checked by spectrophotometry (A_{260}/A_{280} and A_{260}/A_{230}) and by gel electrophoresis.

Probe preparation – Total RNA (10 µg) was added to a mixture of 1 pmol of T20VN and oligo(dT) (18- to 21-mer) primer mix; 0.5 mM each dATP, dGTP, and dTTP; 20.5 µM dCTP; 37.5 µM Cy3- or Cy5-dCTP (NEN Life Sciences, Boston, MA); and 10 mM dithiothreitol in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. The reaction mixture was denatured at 65°C for 5 minutes and incubated at 42°C for 5 minutes, after which 1 µl of RNasin (Promega, Madison, WI) and 200 U of Superscript II reverse transcriptase (Life Technologies/Invitrogen, Carlsbad, CA) were added. The reaction proceeded at 42°C for 1 hour, after which an additional 200 U of Superscript reverse transcriptase were added, and the reaction mixture was incubated at 42°C for 1 h. The reaction was stopped with 5 mM EDTA (pH 8.0) and 0.4 N NaOH and heating at 65°C for 20 min. Finally, acetic acid was added to a final concentration of 0.37 M. Labeled cDNA probes were purified on Qia-quick columns (Qiagen, Valencia, CA). Eluates were pooled, applied to a Microcon-30 filter (Millipore, Bedford, MA), and centrifuged at 18,000 g for 3 min. The membrane unit was vortexed briefly and centrifuged for 60 seconds. The final eluate volume was adjusted to 5 µl with water.

Microarray design and preparation – The *C. albicans* microarray was manufactured by Eurogentec (Seraing, Belgium) in collaboration with the European Galar Fungail Consortium (http://www.pasteur.fr/recherche/unites/Galar_Fungail/). Primers for each of the 6039 putative ORF's in the *C. albicans* genome were designed to amplify a specific region of each ORF, and contained a 15-bp universal sequence at the 5' end to generate a 5' amino-modified product for covalent attachment to aldehyde-coated glass slides. Amplicons (average length of 300 bp) were spotted in duplicate, along with 27 control genes, using a ChipWriter Pro (Virtek Vision Intl., Waterloo, Canada) robotic array printer.

Microarray hybridization – Five µl of each of the Cy3- and Cy5-labeled probes were mixed with 50 µg of heat-denatured salmon sperm DNA, incubated 95°C for 2 minutes, and snap-cooled on ice. The mixture was added to 40 µl of hybridization buffer and applied to the array slides under glass coverslips. Hybridization was performed overnight at 37°C in a humidified chamber (Corning Life Sciences, Acton, MA). To wash the slides, the coverslip was removed, and the slide was incubated at room temperature in 0.2x SSC (1x SSC is 0.15 M NaCl, 0.015 M sodium citrate) plus 0.1% SDS for 5 min with agitation, rinsed at room temperature with 0.2x SSC for 5 min with agitation, and spin-dried at 500 rpm for 5 min. Slides were scanned using a ChipReader microarray scanner (Virtek Vision Intl.).

Data analysis – GenePix 1.0 software (Axon Instruments, Inc.) was used for image analysis and data visualization. The local background values were calculated from the area surrounding each spot and subtracted from the total spot signal values. These adjusted values were used to

determine differential gene expression (Cy3/Cy5 ratio) for each spot. Each of the *C. albicans* genes was present in duplicate on each slide, and the experiment was repeated with the same RNA samples with opposite labels (dye-flip), resulting in four measurements for each gene per sample. Microarray data were normalized with the MARAN web application (http://www.esat.kuleuven.ac.be/maran) using a Lowess-procedure (Yang & Speed, 2002) and a generic model for sequential analysis of variance (ANOVA) (Engelen *et al.*, 2003). Regulated gene candidates were selected when expression level changes were at least two-fold.

Real-time quantitative polymerase chain reaction

Total RNA was extracted from early-log phase cultures, and 2 µg were incubated with 2 U of RQ1 RNAse-free DNAse (Promega, Madison, WI) at 37°C for 30 min. The enzyme was inactivated with 2 µl of RQ1-Stop at 65°C for 10 min. The volume was adjusted to 200 µl with RNAse-free H_2O and the mixture was desalted using Microcon-YM100 (Millipore, Billerica, MA). The eluate, typically about 2.5 µl, was adjusted to 10 µl using RNAse-free dH_2O, supplemented with 2 µg oligo-dT, and incubated at 70°C for 10 min. After cooling on ice, the following components were added: first strand buffer (Life Technologies, Paisley, UK), 3.3 mM dithiothreitol, 40 U RNase Block (Stratagene, La Jolla, CA), 1 mM of each dNTP (Amersham Pharmacia Biotech, Rainham, UK), and 200 U Superscript II reverse transcriptase (Life Technologies, Paisley, UK). The 25 µl mixture was incubated for 1 h at 42°C, and the volume was adjusted to 500 µl. This diluted first-strand cDNA was used as a template in a real-time quantitative polymerase chain reaction (RT Q-PCR), performed in 1x SYBR Green PCR buffer containing ROX as passive reference (Eurogentec, Seraing, Belgium), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 300 nM of each primer and 0.025 U of Hot GoldStar enzyme. SYBR Green was used at a 1:66,000 dilution. The RT Q-PCR was performed in triplicate using the following conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The increase in fluorescence was monitored in real-time using an ABI prism 7700 (Perkin-Elmer Applied Biosystems, Foster City, CA). Primers (Table 1) were designed using the PrimerExpress™ 1.0 software (Perkin-Elmer Applied Biosystems). *IPF3087* (*orf19.7046*) and *DOM34* (*orf19.2419*), or *ACT1* (*orf19.5007*) were used as reference gene(s) for normalization. Specificity of the amplicons generated during the PCR reactions was confirmed by melting curve generation using Dissociation Curves 1.0 (Perkin-Elmer). Gene expression was quantified from threshold cycle values (C_t) at which a statistically significant increase in ROX-normalized fluorescence intensity was first detected. The mean C_t value of three independent RT Q-PCR reactions was normalized to the reference gene(s) within each sample, and the normalized target values of the histatin 5-treated sample and the control sample (untreated) were compared, generating a $\Delta\Delta C_t$ value. The normalized level of target mRNA in the sample relative to the control was expressed as $2^{-\Delta\Delta Ct}$.

Table 1: Forward and reverse primers used in RT Q-PCR experiments for validation of microarray results and for quantification of mRNA levels in the overexpressing strains. *IPF3087* and *DOM34*, or *ACT1*, respectively, were used as reference gene(s) for normalization.

Gene	Primers (5' → 3')
ATP1	GGTGATGTCTCTGCTTATATTCCAACTA
	CCGACGTTAATAGCTGGTCTGATA
CAT2	AAGAACAATTGGCCGCTACAAA
	CCTTGTCACTAGCGAAATCATT TAATC
DDR48	ATGGTTTTCGGTTTCGGTAAAG
	GCCAAATGAAGAGGATCCATAAGA
DOM34	GACATAGACTACATTGCTTCTGATCAAA
	ACCTCAGCGGTGTGGAAACT
IPF18732	CCAGGTAGTCCACCACCAGAAT
	ACGTTCATGATGAGGACCAAATG
IPF3087	ACTGTTCCATCAACAGAGGTTTCA
	CACCATTGCCAAATACATCTAATGA
IPF3964	TGACAAGTTTAGCTGACGGAAAAT
	GTTAAGGAATCTTTTTTGGATTCAATCT
IPF4119.5	GCCATTCACGTGTTCCGATAT
	AATGCGTGAATGATACCTGGAATA
PGA3	GCAATGCTAACACAACTATGTCAAACT
	GCCATTACCTTGAGGAGCAGTAGA
PMC1	ACTCCTATGCAAGTACGTTTGAATGA
	CACAGAATCTAATGAATAAAACCACAAAT
POR1	CTGCTGATTTAGTTGTTGCT CATGAT
	GCGTAACCAACACCAACAGAATATT
RTA2	ACTTCCACACCTACATCCACTACATT
	TCAGTGGTGGCTTGAGAAATCA
SNF31	TTCCGCTGCCAGAGGTATTT
	CGTAGTCCATAGACAATACACCAGAAA
ACT1	GCTGAACGTATGCAAAAGGAAATT
	AGAACCACCAATCCAGACAGAGTATT
GPR1	CTTTGTGTCAATGAAAGATCAATGG
	TTGTACCAGAT GCATTATGGCTATG
IPF2973	CATTTATGCATTTAGTGAGACCAAATG
	TTGTGAAATGATTTCTAAAATTGTCAACA
TRX1	TGGTTCACGTTGTCACTGAAGTT
	CATTTTACATGGACCACACCAAGT

Plasmid construction

A 633 bp fragment of the *Candida ARG5,6* gene was excised from pGAL1PHAtHst5ARGT (K. De Smet, unpublished) and cloned into a *Pvu*II-opened, dephosphorylated pACT1P(B)yEGFP3TU-1 vector (M. Logghe, unpublished) to produce pACT1P(B)yEGFP3TUdARGs-1. Genes of interest were amplified from genomic DNA (cDNA for ATP1) using the primers shown in Table 2, *Pfu* DNA polymerase (Stratagene Cloning Systems, La Jolla, CA) and standard PCR conditions. PCR products were cloned in pCR-BluntII-TOPO (Stratagene Cloning Systems, La Jolla, CA) and sequenced. Genes with the correct sequence were excised from the TOPO-vector using *Eco*RI, and sticky ends were blunted. *GPR1* was excised from Ycplac33/CaGPR1wrong (P. Van Dijck, unpublished) using *Nru*I and *Not*I, and sticky ends were blunted. *TRX1* was excised from pACT1P:sTRX (K. De Smet, unpublished) using *Bam*HI and *Asp*I, and sticky ends were blunted. *IPF2973* was excised

from pRM2MAL2P2000SN clone M4 using *Sfi*I and *Not*I, and sticky ends were blunted. The expression vector pACT1P(B)yEGFP3TUdARGs-1 was opened using *Bam*HI and *Asp*I, sticky ends were blunted, and the vector was dephosphorylated. The excised genes were ligated into the opened vector in a blunt-blunt ligation. Correct integration (sense direction) was checked using restriction digests. All expression plasmids are listed in Table 3.

A control vector (pACT1PUdARGs, empty vector) was constructed by self-ligation of the *Bam*HI-*Asp*I opened and blunted pACT1P(B)yEGFP3TUdARGs-1 (Fig. 1b).

Table 2: Forward and reverse primers used for isolating *C. albicans* genes of interest. Start and stop codons are in bold; restriction sites are underlined; K, T+G; Y, C+T; W, A+T.

Gene	Primers (5' → 3')
ATP1	**ATG**TTGKCYCGTWCTGC
	TTATTAGAAAGAAGCAACG
CAT2	CATA GGCCAAAAAGGCC**ATG**TTTAAATTTAGAATTCCACAAC
	CGCGGATCC**TTA**CAATTTGGCCTTTGGTTCAG
DDR48	CCCAAGCTT**ATG**GTTTTCGGTTTCGGTAAAGACG
	TCCCCCCGGG**TTA**ATAGTCAGAAGATCCATAGGAGTC
IPF18732	CCCAAGCTT**ATG**TTTCTTCATAATTTATTAAATAAACATC
	CGCGGATCC**CTA**ACTTATTTGCAACCTAATAGAATC
IPF3964	CCCAAGCTT**ATG**CCAGTTCAACCAAGAGACC
	CGCGGATCC**TCA**AGCTAAGCTCAAAATAAACG
IPF4119.5	CCCAAGCTT**ATG**CCATTCACGTGTTCCGATATC
	CGCGGATCC**CTA**GTACTTTAGAATGACGTACAATG
PGA3	CCCAAGCTT**ATG**AAGTATTTGTCCATTTTCTTAC
	CGCGGATCC**TTA**AATCAAGGCAGCAATGACACC
PMC1	CATA GGCCAAAAAGGCC**ATG**GCTCCAAGAACACCAGCCG
	*CGCGGATCC***CTA**TTCCTTTGATTCTTTTAAATCGG
POR1	CATA GGCCAAAAAGGCC**ATG**GCTCCAGCTGCTTATTCTG
	CGCGGATCC**TTA**AGCAGCAAAAGACAAAGAGAAAC
RTA2	CATA GGCCAAAAAGGCC**ATG**AGTGAAATCTTGAATTATTTATCTTCG
	CGCGGATCC**TCA**TAACTTACTATTTAAACAATGTATGTC
SNF31	CATA GGCCAAAAAGGCC**ATG**AGTGCAAATATCCAAGCTC
	CGCGGATCC**TTA**AACGGAATTTTCATCAACTTC

Generation of the expression strains

C. albicans CAI-4 was transformed with the expression plasmids using a modified version (M. Logghe, unpublished) of the spheroplasting protocol (Herreros *et al.*, 1992). The plasmids were linearized to allow direct integration in the *ARG5,6* gene, the *ACT1* promoter or the *MAL2* terminator. Genomic DNA was prepared from different transformants (DNA Pure Yeast Genomic kit of CPG Inc., Lincoln Park, NJ). Correct integration in the genome was confirmed by standard PCR on 500 ng genomic DNA, using the primers 5'-TTTTACAACGTCGTGACTGG-3' and 5'-AGTGGCAACAATAACAACACG-3' for

ARG5,6; 5'-TGGATAACCGTATTACCGCC-3' and 5'-TGAGAACAGCGACCGAAAGC-3' for *ACT1* promoter; 5'-CCTTTTCTAATTTTCACTCCTGG-3' and 5'-GAAATGAAATGCAAAGACTGC-3' for *MAL2* terminator. Expression of the transformed genes was confirmed in several transformants using RT Q-PCR and *ACT1* as reference gene, as described above.

Growth assay

Cultures were grown overnight in LYM medium, diluted in the same medium and grown to early-log phase. Cells were washed and resuspended in H_2O at 2 x 10^7 cells/ml. Two µl of cell suspension containing $4x10^4$ cells were inoculated in 200 µl LYM medium in the presence or absence of histatin 5 in a Honeywell plate. They were grown at 30°C for 72 h with intermittent shaking (10 sec every 10 min). Growth was assayed by measuring optical densities every hour on a Bioscreen C (Labsystems, Helsinki, Finland).

Table 3: Representation of steps in constructing expression plasmids for the overexpression of genes of interest in *C. albicans*. Histatin 5-responsive genes from microarray analysis, validated with RT Q-PCR (*) were isolated using PCR and cloned into the pCR-BluntII-TOPO vector, fragments were sequence verified. Genes isolated using library screening and other genes of interest were cloned from other plasmids (§). pACT1PdARGs is the empty vector that is used as a control (c). na, not applicable.

Gene	PCR	Plasmid	Sequence	Expression plasmids
*ATP1**	/	/	/	/
*CAT2**	OK	pCR-BluntII-TOPO-CaCAT2	OK	pACT1PUdARGs:CaCAT2
*DDR48**	OK	pCR-BluntII-TOPO-CaDDR48	OK	pACT1PUdARGs:CaDDR48
*IPF18732**	OK	pCR-BluntII-TOPO-CaIPF18732	/	/
*IPF3964**	OK	pCR-BluntII-TOPO-Ca3964	OK	pACT1PUdARGs:CaIPF3964
*IPF4119.5**	OK	pCR-BluntII-TOPO-Ca4119.5	OK	/
*PGA3**	OK	pCR-BluntII-TOPO-CaPGA3	OK	pACT1PUdARGs:CaPGA3
*PMC1**	/	/	/	/
*POR1**	OK	pCR-BluntII-TOPO-CaPOR1	OK	pACT1PUdARGs:CaPOR1
*RTA2**	/	/	/	/
*SNF31**	OK	pCR-BluntII-TOPO-CaSNF31	OK	pACT1PUdARGs:CaSNF31
TRX1§	na	pACT1P:sTRX	na	pACT1PUdARGs:CaTRX1
IPF2973§	na	pRM2MAL2P2000SN cl.M4	na	pACT1PUdARGs:CaIPF2973
GPR1§	na	Ycplac33/CaGPR1wrong	na	pACT1PUdARGs:CaGPR1
\C	na	na	na	pACT1PUdARGs

Results

Library screening

Library construction – We constructed a *C. albicans* replicative vector, pRM2MAL2P2000SN (Fig. 1a), for directional cloning of *C. albicans* cDNA with a *Sfi*I adapter at the 5' end and a *Not*I adapter at the 3' end. The vector contains the inducible *CaMAL2* promoter, a *Sfi*I-*Not*I 2000 bp stuffer fragment, *C. albicans URA3* and *MET15* selection markers, a *C. albicans WH11* transcription terminator, a *S. cerevisiae* cytochrome C transcription terminator, three *C. albicans* autonomous replicating sequences (ARS), and elements to allow autonomous replication and selection in *E. coli*. A *C. albicans* cDNA library, suitable for inducible sense expression in *C. albicans*, was ligated in this vector and the library was transformed to *C. albicans* CAI-4.

Histatin 5 treatment – *C. albicans* CAI-4 cells transformed with the *C. albicans* cDNA sense library were grown in SDC to early-log phase. The culture was split; one part was induced in medium containing maltose, and the other part in medium containing sucrose. After induction each culture was treated with 100 µM histatin 5 for 2 h, as described in Materials and Methods, to enrich for cells that are more resistant to histatin 5-induced cell death. After the first treatment, 3% of cells induced on SMC and 10% of those induced on SSC survived. After the third round of treatment, cell survival of SMC-induced cells was 3.5%, and for SSC-induced cells it was 18%. The enrichment for more resistant cells was minimal in the SMC-induced culture (0.5%), but substantial in the SSC-induced culture (8%). All surviving cells were plated on YPD agar. The cells induced on medium with maltose formed 113 colonies (M1-M113), and those induced on medium with sucrose formed 79 colonies (S1-S79).

Individual survival assays – Some survivor colonies (M1-M34 and S1-S10) were tested for their sensitivity to histatin 5-induced cell death. Five transformants induced in SMC (M4, M5, M6, M8 and M27) and four transformants induced in SSC (S6, S7, S8, S10) were more resistant than the wild type strain (CAI-4) (Fig. 2).

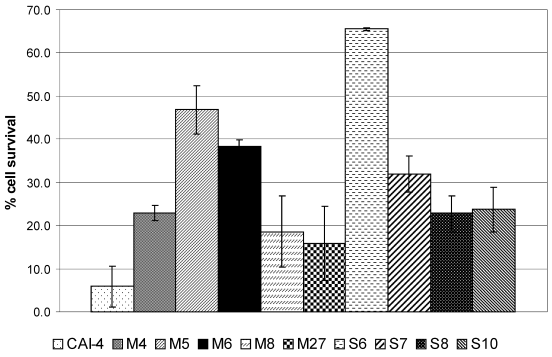

Figure 2: Individual survival assays of the colonies remaining after library screening; these colonies were more resistant than the wild type. Cells were treated with 100 µM histatin 5 for 2 h. Samples were diluted, plated on YPD agar and incubated for 48 h at 30 °C. The data shown are means and standard deviations of at least two experiments. M4, M5, M6, M8 and M27: transformants induced in SMC. S6, S7, S8 and S10: transformants induced in SSC.

DNA of these strains was isolated and transformed to *E. coli* to prepare plasmid DNA, and cDNA inserts were sequenced. Only two inserts contained a full-length gene. The insert of strain M4 contained *IPF2973* (*orf19.4283*), a gene of unknown function. The IPF2973 protein contains a Mov34 domain, which is found in the N-terminus of the proteasome regulatory subunits, in eukaryotic initiation factor 3 (eIF3) subunits, and in regulators of transcription factors, but its function is unknown. A protein homology search showed that IPF2973 shares homology with a regulatory subunit of the 26S proteasome of several pathogenic yeast species. A nucleotide homology search showed that *IPF2973* has 29% sequence similarity to the *S. cerevisiae GPR1*. The insert of strain M5 contained *TRX1* (*orf19.7611*), a thioredoxin. The plasmids isolated from the other strains contained no open reading frames (M6, S6) or only a part of a gene (S7: less then 1/3 of an ABC transporter gene; S8: a small part of cytochrome C peroxidase).

Construction of expression vector – We wanted to study the effects of overexpressing *TRX1* and *IPF2973* on histatin 5-induced cell death. Furthermore, because IPF2973 is homologous to *S. cerevisiae* Gpr1, we also decided to study *Candida* Gpr1 (*orf19.1944*). We constructed a new integrative expression vector (pACT1P(B)yEGFP3TUdARGs-1) and cloned those 3 genes in it.

Expression analysis – pACT1PUdARGs:CaIPF2973 was linearized in the *ACT1* promoter, and pACT1PUdARGs:CaTRX1 and pACT1PUdARGs:CaGPR1 in the *ARG5,6* fragment. The linearized plasmids were transformed to *C. albicans* CAI-4. Transformants were selected on uridine-free minimal medium. Correct integration was confirmed by PCR (data not shown). As no antibodies were available against most of the overexpressed proteins, mRNA levels were determined using RT Q-PCR and compared with those of the control strain (CAI-4 transformed with pACT1PUdARGs). Three transformants of each strain were grown in selective medium and RNA was extracted from early-log phase cultures. The RNA samples were DNAse-treated and cDNA was synthesized. The cDNA samples were used for RT Q-PCR analysis. The TRX1 mRNA levels in transformants 1 and 2 were only slightly higher compared to the control strain, and no overexpression could be detected in transformant 3 (Fig 3a). IPF2973 mRNA levels were clearly increased in all the IPF2973-overexpressing strains (Fig. 3b); expression levels were 9, 127 en 66 times higher than in the control strain. The three GPR1-overexpressing strains also showed clearly increased levels of GPR1 mRNA (Fig. 3c); expression levels were between 3.1 and 4.3 times higher than in the control strains.

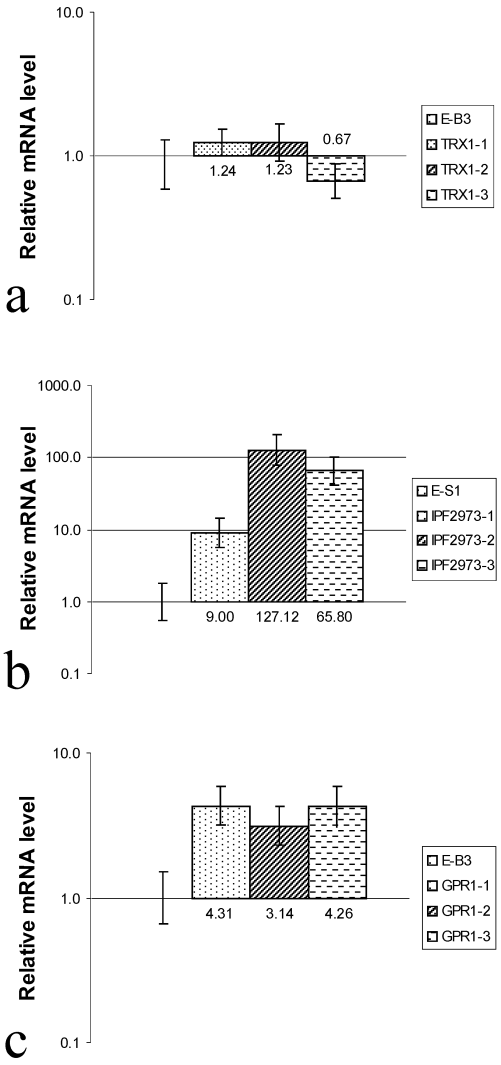

Figure 3: Quantification of mRNA levels of overexpressing strains using RT Q-PCR. Three transformants of each strain were analysed and each reaction was run in triplicate. (a) *TRX1*-overexpressing strain, (b) *IPF2973*-overexpressing strain and (c) *GPR1*-overexpressing strain. E-B3 and E-S1: control strains, transformed with empty plasmid (pACT1PdARGs). mRNA levels of the control strains are set at 1.

Growth assay of overexpressing strains – To study whether overexpression of these genes has an influence on histatin 5 sensitivity, growth assays were performed in its presence and in its absence. The growth of two IPF2973 transformants (1 and 2), one GPR1 (3) and one TRX1 transformant (1) in LYM medium was monitored for 72 h (Fig. 4). All transformants showed increased resistance to histatin 5 compared to the control strains, indicating that overexpression of these three genes has an influence on histatin 5 sensitivity. Although *TRX1* was only slightly overexpressed in transformant 1, its effect was evident in the growth assay (Fig. 4a). The high level of overexpression of IPF2973 in strain IPF2973-2 (mRNA levels 127 times higher than in the control strain) results in a highly resistant strain (Fig. 4b). The other IPF2973 overexpressing strain that was tested (IPF2973-1) was also more resistant, although the resistance was somewhat lower than that of IPF2973-2 (data not shown), probably because of the lower level of overexpression of the gene (mRNA levels 9 times higher than in the control strain). The GPR1-overexpressing strain was also more resistant than the control strain. The

GPR1-overexpressing strain is the only one whose growth was impaired in the absence of histatin 5 (Fig. 4c).

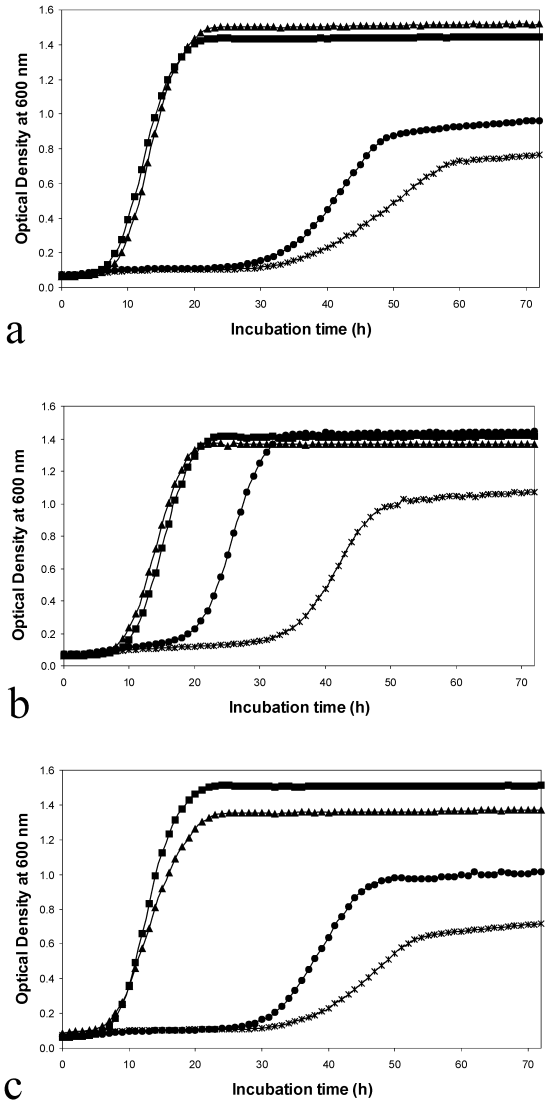

Figure 4: The effect of overexpression of the genes isolated by library screening on growth in the absence and presence of 7.5 µM histatin 5. (a) *TRX1*-overexpressing strain, (b) *IPF2973*-overexpressing strain and (c) *GPR1*-overexpressing strain. Wild-type in the absence of histatin 5 (squares), wild-type in the presence of 7.5 µM histatin 5 (crosses), overexpressing strain in the absence of histatin 5 (triangles) and overexpressing strain in the presence of 7.5 µM histatin 5 (circles). Growth curves are representative of at least 3 independent experiments.

Integration of the expression plasmids by homologous recombination in the *ARG5,6* gene of *C. albicans* using only the middle part of this gene as homologous sequence knocks out one allele. However, this had little or no effect on the growth of the control strains (E-H3 and E-B3) or on their sensitivity to histatin 5. Integration in the *ACT1* promoter causes duplication of the *ACT1* promoter, but this too has little or no consequences for this strain (E-S1) in terms of growth or sensitivity to histatin 5 (Fig. 5).

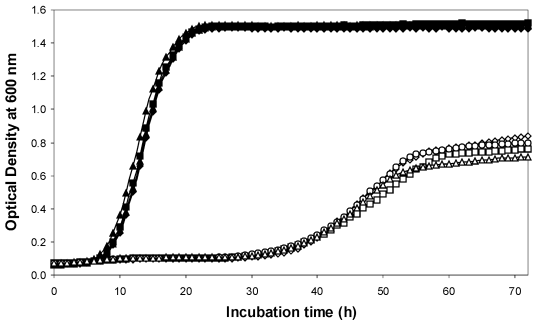

Figure 5: The effect of integration of pACT1PdARGs in different loci of the genome on growth in the absence (closed symbols) and presence of 7.5 µM (open symbols) histatin 5. Wild-type (diamonds); integration in *ARG5,6*, plasmid digested with *Hpa*I (squares) or *Bsr*GI (triangles); and integration in *ACT1* promoter (circles).

Growth assay of GPR1 deletion strain - Overexpression of *GPR1* makes the cells more resistant to histatin 5-induced cell death. Molecular targets for the development of new antifungal therapeutics are those functions whose knock-out sensitizes the cells to histatin 5. To determine whether knocking out *GPR1* heightens this sensitivity, we tested these knockout strains in a growth assay. The parental strains CAI-4 and the knockout strains, LDR1 (*gpr1/GPR1*) and LDR8 (*gpr1/gpr1*), were grown at 30°C for 72 h in LYM medium in the presence and absence of histatin 5. Optical densities were measured every hour and growth curves were generated (Fig. 6). Both strains were more susceptible to histatin 5-induced cell death than the parental strain. Grown in the presence of 2.5 µM histatin 5, both knockout strains were more sensitive than the wild-type strain. When histatin 5 concentration is raised to 5 µM, a difference in susceptibility between the knockout strains is observed: knocking out both *GPR1* alleles makes the cells more sensitive than the heterozygote strain. At a histatin 5 concentration of 7.5 µM, the knockout strains do not grow, while the parental strain did. It is clear that Gpr1 influences growth in the presence of histatin 5: overexpression of Gpr1 makes the cells more resistant to histatin 5, whereas deletion of *GPR1* makes them more susceptible.

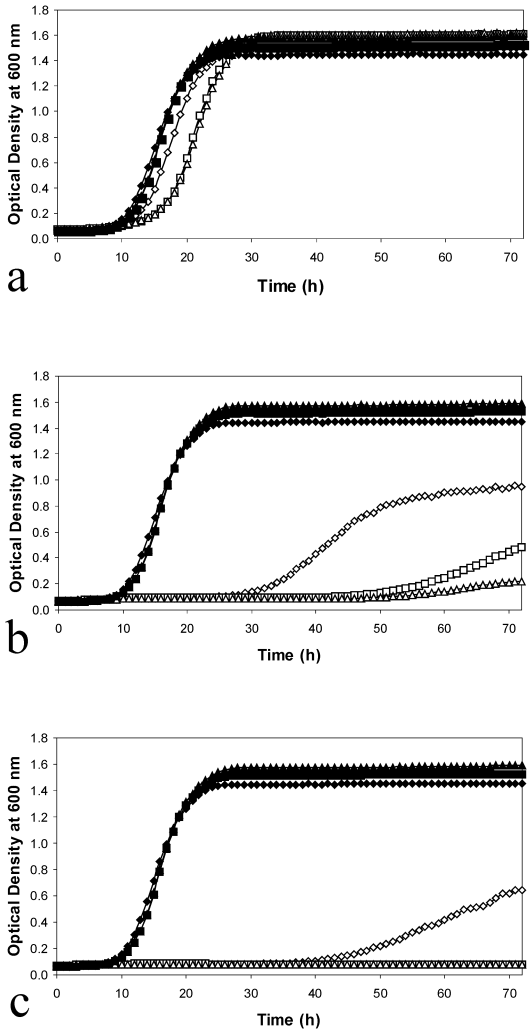

Figure 6: The effect of deleting *GPR1* on growth in the absence (closed symbols) or presence (open symbols) of 2.5 µM (a), 5 µM (b) and 7.5 µM (c) histatin 5. Wild-type (diamonds); LDR1, single *GPR1* knockout (squares) and LDR8, double *GPR1* knockout (triangles). Growth curves are representative of at least 3 independent experiments.

Growth assay of GPA2 deletion strain - In *S. cerevisiae* the membrane bound protein, Gpr1, interacts with Gpa2. These two proteins form a G-protein-coupled receptor system and are involved in extracellular glucose/sucrose detection, pseudohyphal differentiation, and stress resistance (Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Lemaire *et al.*, 2004). It was also shown recently that in *C. albicans* Gpr1 interacts with Gpa2 and signaling goes through the cAMP-PKA pathway, where it is involved in hypha formation on solid medium. Contrary to the situation in *S. cerevisiae*, Cdc25 and Ras1 seem to be involved in the glucose-induced cAMP increase, not Gpr1 (Maidan *et al.*, 2005). To check whether Gpa2 is also involved in cell death induced by histatin 5, we analyzed the growth properties of the double *GPA2* deletion strain (Fig. 7). Histatin 5 did not impair the growth of this *GPA2* deletion strain, which grew somewhat better than the control strain. This phenotype does not correspond to that of the *GPR1* deletion strain, indicating that Gpa2 can function independent of Gpr1 and vice versa. Whereas it is clear that Gpr1 is upstream of the cAMP-PKA pathway, it has been suggested that Gpa2

may also be upstream of the Mitogen Activated Protein Kinase pathway (Sanchez-Martinez & Perez-Martin, 2002).

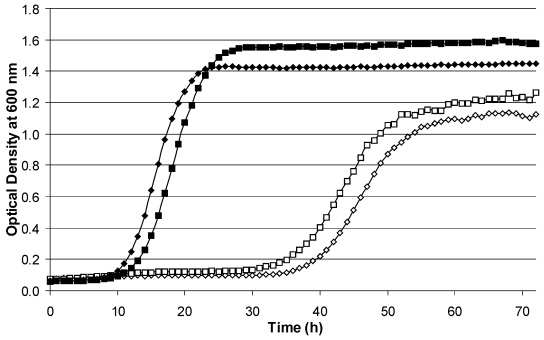

Figure 7: The effect of deletion of *GPA2* on growth in the absence (closed symbols) or presence of 7.5 µM (open symbols) histatin 5. Wild-type (diamonds) and NM29, double *GPA2* knockout (squares).

Expression profiling

Microarray analysis - RNA was extracted from a *C. albicans* CAI-4 culture grown to early-log phase and treated for 40 min with 25 µM histatin 5, which caused 30% cell mortality. Control RNA was extracted from cells from the same initial culture incubated for 40 min in the same conditions but without histatin 5. The quality of the RNA was checked by spectrophotometry. cDNA synthesis, labeling with Cy3 and Cy5, hybridization with microarray slides and scanning of the slides were performed as described in Materials and Methods. The *C. albicans* microarray consisted of 6039 PCR-amplicons of open reading frames, covering nearly 98% of the annotated *C. albicans* genes. Each of the *C. albicans* genes was present in duplicate on each slide, and the experiment was repeated with the same RNA samples with opposite labels (dye-flip), resulting in four measurements for each gene per sample. Microarray data were normalized with the MARAN web application (http://www.esat.kuleuven.ac.be/maran) using a Lowess-procedure (Yang & Speed, 2002) and a generic model for sequential analysis of variance (ANOVA) (Engelen *et al.*, 2003). Sixty genes were found to be differentially regulated by a factor of at least 2 (Table 4). Ten of these genes were upregulated, and fifty were downregulated.

Table 4: Histatin 5-responsive genes whose expression is changed at least 2-fold after a 40-minute treatment with 25 µM histatin 5. *, genes selected for validation in RT Q-PCR experiment and for overexpression studies. CGD: Standard name in Candida Genome Database (http://www.candidagenome.org/).

Gene		**Description**	**Ratio**
CandidaDB	ORF19 annotation (CGD)	CandidaDB	
Amino acid metabolism (5)			
UGA12.3f	orf19.803	4-aminobutyrate aminotransferase, 3-prime end	0.33
UGA12.5f	orf19.802	4-aminobutyrate aminotransferase, 5-prime end	0.39
DUR1,2	orf19.780	urea amidolyase	0.42
UGA2	orf19.345	succinate-semialdehyde dehydrogenase [NADP+]	0.49
ARG1	orf19.7469 (*ARG1*)	argininosuccinate synthetase	0.49
Carbohydrate metabolism (18)			
ADH2	orf19.5113 (*ADH2*)	alcohol hydrogenase I	0.24
PYC2.exon2	orf19.789 (*PYC2*)	pyruvate carboxylase 2, exon 2	0.30
INO1	orf19.7585 (*INO1*)	myo-inositol-1-phosphate synthase	0.31
IFE2	orf19.5288 (*IFE2*)	alcohol dehydrogenase	0.33
*SNF31**	orf19.7094 (*HGT12*)	high-affinity glucose transporter	0.33
HGT12	orf19.3668 (*HGT2*)	hexose transporter	0.34
ACS1	orf19.1743 (*ACS1*)	acetyl-coenzyme-A synthetase	0.34
MLS1	orf19.4833 (*MLS1*)	malate synthase	0.37
IPF2852	orf19.7288	putative acetyl-coenzyme-A dehydrogenase	0.38
ADH1	orf19.3997 (*ADH1*)	alcohol dehydrogenase III	0.39
ICL1	orf19.6844 (*ICL1*)	isocitrate lyase	0.41
HGT11	orf19.4527 (*HGT1*)	hexose transporter	0.42
IDP2	orf19.3733 (*IDP2*)	isocitrate dehydrogenase	0.43
FBA1	orf19.4618 (*FBA1*)	fructose-bisphosphate aldolase	0.44
ACS2	orf19.1064 (*ACS2*)	acetyl-coenzyme-A synthetase	0.45
FBP1	orf19.6178 (*FBP1*)	fructose-1,6-bisphosphatase	0.47
IPF15957	orf19.1105.2 (*PGA56*)	regulator of sorbose utilization	0.47
CIT1.exon2	orf19.4393 (*CIT1*)	citrate synthase, exon 2	0.48
Degradation (1)			
UBI4	orf19.6771 (*UBI4*)	polyubiquitin	0.50
Energy generation (3)			
ALD5	orf19.5806 (*ALD5*)	aldehyde dehydrogenase (NAD+)	0.40
ARD8	orf19.6322 (*ARD*)	D-arabinitol dehydrogenase	0.43
ATP1.exon3*	orf19.6854 (*ATP1*)	F1F0-ATPase complex, F1 alpha subunit, exon 3	0.50
Lipid-fatty acid metabolism (10)			
*RTA2**	orf19.24 (*RTA2*)	phospholipid-translocating ATPase activity stress associated	2.50
POX4	orf19.1652 (*POX1-3*)	peroxisomal fatty acyl-CoA oxidase	0.34
POT11	orf19.7520 (*POT1*)	peroxysomal 3-ketoacyl-CoA thiolase A	0.36
POT1	orf19.2046 (*POT1-2*)	acetyl-CoA C-acyltransferase	0.38
POT12	orf19.1704 (*FOX3*)	peroxysomal 3-ketoacyl-CoA thiolase B	0.40
*CAT2**	orf19.4591 (*CTN2*)	carnitine O-acetyltransferase	0.42
FOX2	orf19.1809 (*FOX2*)	hydratase-dehydrogenase-epimerase	0.43
IPF15679	orf19.1709	lipid transfer protein	0.45
PXP5	orf19.5723 (*POX1*)	acyl-coenzyme A oxidase I	0.46
FAA21	orf19.272 (*FAA2-2*)	long-chain-fatty-acid-CoA ligase	0.49

Table 4: Continued.

Protein modification (1)			
PRB1	orf19.7196 (*PRB1*)	protease B, vacuolar	0.47
Stress response (4)			
*PGA3**	orf19.2060 (*SOD5*)	similar to superoxide dismutase	4.17
*DDR48**	orf19.4082 (*DDR48*)	stress protein	3.85
CTA1	orf19.6229 (*CAT1*)	catalase A	0.39
AOX2	orf19.4773 (*AOX2*)	alternative oxidase	0.49
Transcription (1)			
TEF1	orf19.1435 (*TEF2-2*)	translation elongation factor eEF1 alpha-A chain	0.44
Transport (6)			
*PMC1**	orf19.1727	Ca2+-transporting P-type ATPase	2.03
IPF7493	orf19.4682 (*HGT17*)	putative permease	0.38
*POR1**	orf19.1042 (*POR1*)	mitochondrial outer membrane porin (VDAC 1)	0.44
IFC1	orf19.3746 (*IFC1*)	oligopeptide transporter	0.44
IFC4	orf19.2292 (*OPT4*)	oligopeptide transporter	0.47
OPT2.53f	orf19.2847.1	oligo peptide transporter, internal fragment	0.48
Unknown (11)			
*IPF18732**	orf19.711	sequence similarity to histidine-rich glycoprotein precursor of *Plasmodium lophurae*	3.14
*IPF3964**	orf19.675	unknown function	3.05
IPF4443	orf19.4706	unknown function	2.55
IPF7109	orf19.7350	unknown function	2.42
*IPF4119.5**	orf19.2030	unknown function	2.19
IPF5192	orf19.3615	unknown function	2.07
AMO2	orf19.3152 (*AMO2*)	similar to amine oxidase	0.32
IPF263.3	orf19.3263	member of the FRP family of proteins related to *Y. lipolytica* glyoxylate pathway regulator Gpr1p and *S. cerevisiae* Fun34p	0.33
FUN34.5eoc	orf19.6169.2	unknown function, 5-prime end	0.34
FRP1	orf19.2496 (*FUN34*)	member of the FRP family of proteins related to *Y. lipolytica* glyoxylate pathway regulator Gpr1p and *S. cerevisiae* Fun34p	0.38
IPF6257	orf19.1461	unknown function	0.49

Real time quantitative PCR - The levels of expression of eleven histatin 5 responsive genes, marked in Table 4 with an asterisk, were verified by real-time quantitative PCR relative to the genes *IPF3087* (*orf19.7046*) and *DOM34* (*orf19.2419*), whose transcript levels were unaffected upon histatin 5 treatment (data not shown). It was confirmed that all of the eleven genes have the same histatin 5 transcriptional regulation (Table 5) compared to the microarray analysis, emphasizing the reliability of the microarray analysis.

Table 5: Comparison of expression levels of histatin 5-responsive genes, obtained using microarray analysis or RT Q-PCR.

Gene	Microarray	RT Q-PCR
PGA3	4.17	29.86
DDR48	3.85	5.50
IPF18732	3.14	5.21
IPF3964	3.05	8.40
RTA2	2.50	6.73
IPF4119.5	2.19	2.60
PMC1	2.03	6.73
SNF31	0.33	0.19
CAT2	0.42	0.45
POR1	0.44	0.54
ATP1.exon3	0.50	0.58

Construction of overexpression plasmids - These eleven histatin 5 responsive genes were chosen for further analysis, and the influence of overexpression of these genes on histatin 5-induced cell death was determined. Using standard PCR conditions and the primers shown in Table 2, we were able to isolate eight genes. The other three genes - *ATP1* (*orf19.6854*, a gene with an exon-intron structure), *PMC1* (*orf19.1727*) and *RTA2* (*orf19.24*) – could not be amplified although several PCR conditions were tried. The eight isolated genes were cloned into the pCR-BluntII-TOPO vector and verified by sequencing. The sequences of seven of the eight genes were correct, and only the sequence of *IPF18732* (*orf19.711*) was incorrect in all the clones that were checked. Though we could not clone *IPF4119.5* (*orf19.2030*) in pACT1PUdARGs, the other genes could be cloned (Table 3). These six plasmids were transformed to *C. albicans* CAI-4 and transformants were grown on selective minimal medium. Three transformants of each overexpressing strain were grown to early-log phase and analyzed by RT Q-PCR for steady-state mRNA levels of the genes that are overexpressed (Fig. 8).

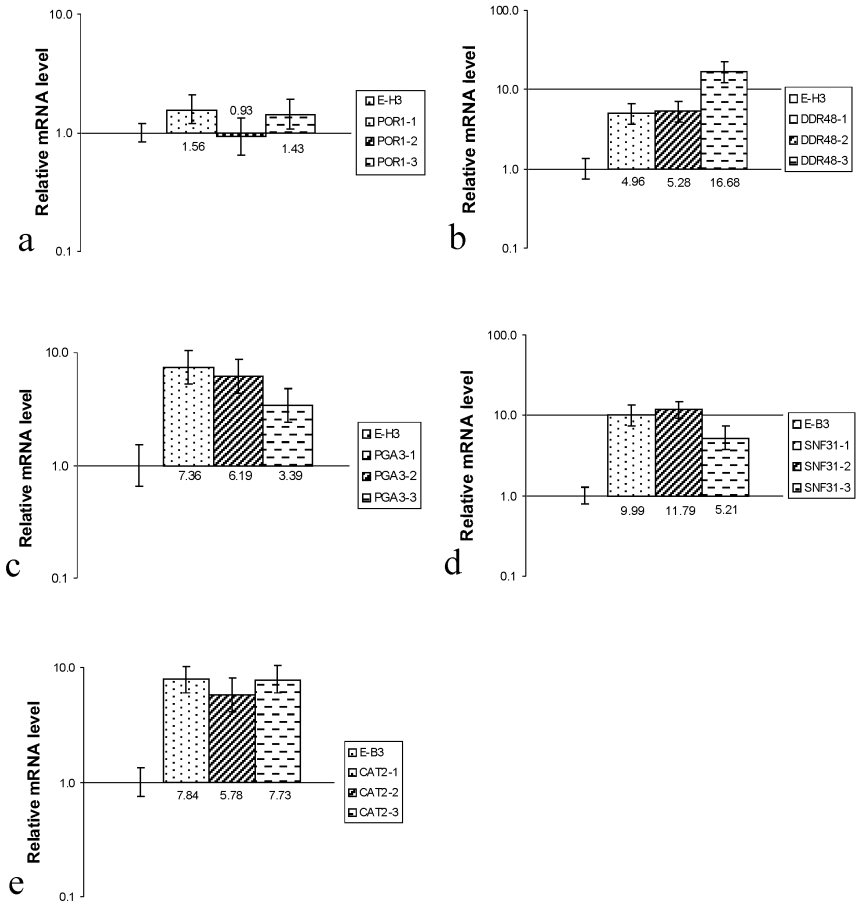

Figure 8: Quantification of mRNA levels of overexpressing strains using RT Q-PCR. Three transformants of each strain were analyzed, each reaction was run in triplicate. (a) *POR1*-overexpressing strain, (b) *DDR48*-overexpressing strain, (c) *PGA3*-overexpressing strain, (d) *SNF31*-overexpressing strain and (e) *CAT2*-overexpressing strain. E-B3 and E-H3: control strains transformed with empty plasmid (pACT1PdARGs). mRNA levels of the control strains are set at 1.

Overexpression was successful in *DDR48* (*orf19.4082*), *SNF31* (*orf19.7094*), *PGA3* (*orf19.2060*) and *CAT2* (*orf19.4591*), with mRNA levels ranging from 3.4 to 16.7 times higher than in the control strains. *POR1* (*orf19.1042*) was only slightly overexpressed in 2 of the 3 transformants analyzed (1.6 and 1.4 times higher mRNA levels). In the transformants overexpressing *IPF3964* (*orf19.675*), no elevation in mRNA levels could be detected compared to the control strain (data not shown).

Growth assays - Strains overexpressing *POR1*, *DDR48*, *SNF31*, *PGA3* and *CAT2* were further analyzed in growth assay experiments in LYM medium with 5 and 7.5 µM histatin 5 (Fig. 9).

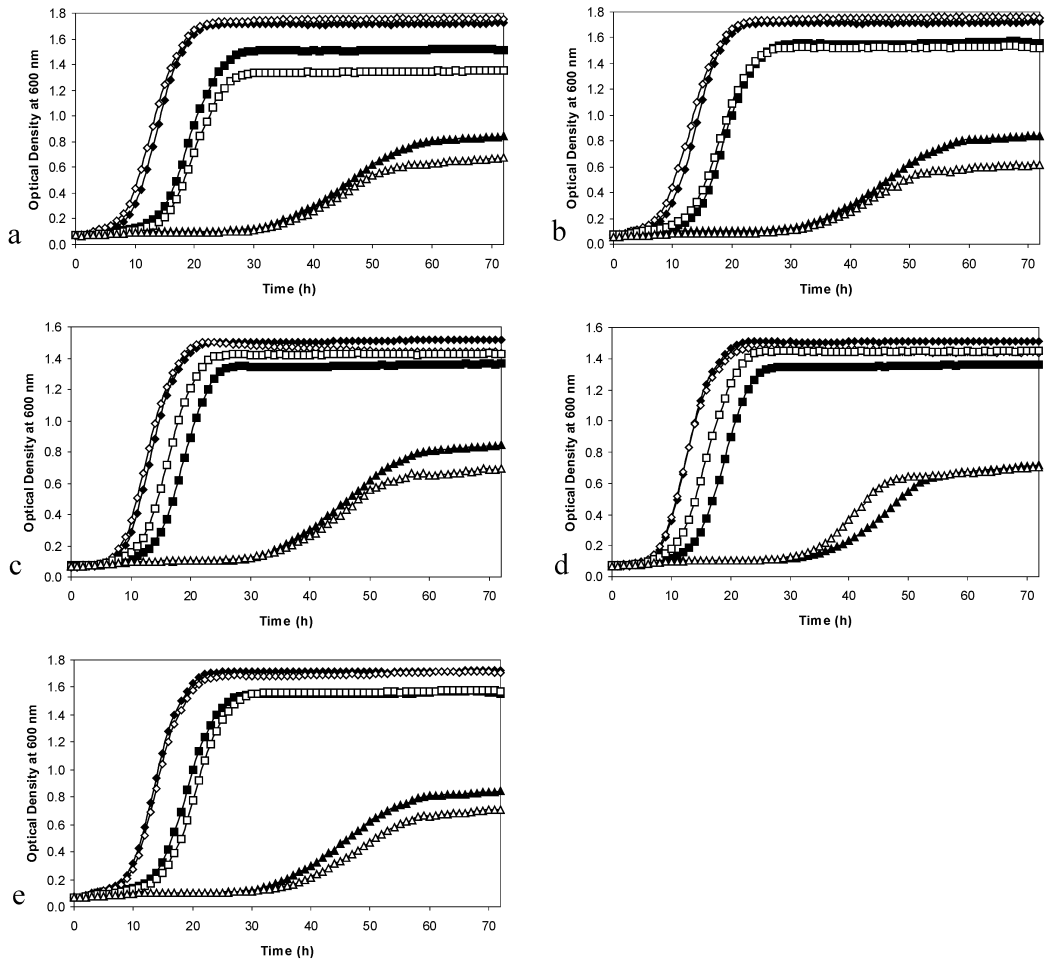

Figure 9: The effect of overexpression of the genes isolated by library screening on growth in the absence (diamonds) and presence of 5 µM (squares) and 7.5 µM (triangles) histatin 5. (a) *POR1*-overexpressing strain, (b) *DDR48*-overexpressing strain, (c) *PGA3*-overexpressing strain, (d) *SNF31*-overexpressing strain and (e) *CAT2*-overexpressing strain. Wild-type (closed symbols) and overexpressing strain (open symbols). Growth curves are representative of at least 3 independent experiments.

Although strains overexpressing *POR1* had only slightly higher mRNA levels than the parental strain, an influence on growth was visible when these strains were grown in the presence of histatin 5. Strains overexpressing *POR1* were more sensitive to histatin 5 than the control strain. When the other strains were grown in the presence of histatin 5, there was also an influence on growth. Strains overexpressing *DDR48* were more susceptible to histatin 5 when grown in medium supplemented with 7.5 µM histatin 5. No difference in growth was observed when the growth medium was supplemented with 5 µM histatin 5. Overexpression of *PGA3* makes the cells more resistant to 5 µM histatin 5, but at 7.5 µM the resistant phenotype disappears. The expression levels of transformants 1 and 2 are about 7 times higher than that of the control

strain, whereas transformant 3 has a mRNA level that is half of the other transformants. This difference in expression level was manifested in the growth assay: growth of transformant 3 in the presence of histatin 5 was the same as that of the control strain (data not shown). The level of overexpression in this transformant was probably not enough to affect growth rate. Overexpression of *SNF31* increased resistance to 5 µM histatin 5, but in the presence of 7.5 µM only transformant 2 still showed a slightly more resistant phenotype. Differences in phenotype could not be detected when the CAT2-strain was grown in the presence of 5 µM histatin 5. The transformants were somewhat more sensitive to 7.5 µM histatin 5.

Discussion

In this study we tried to identify genes involved in histatin 5-induced cell death in the pathogenic yeast *C. albicans*, which might help to resolve the mechanism of action of histatin 5. Furthermore, functions involved in this cell death process can be used as targets for the development of novel antifungal compounds. We sought to identify these functions by screening an overexpression library and by microarray expression profiling. A *C. albicans* cDNA library was constructed and cloned, behind the inducible *MAL2* promoter, into a new replicative vector. *C. albicans* cells, transformed with this overexpression library, were treated with histatin 5 and the cDNA inserts of the surviving cells were sequenced. Two inserts corresponded to full-length genes: *TRX1* (*orf19.7611*), a thioredoxine and *IPF2973* (*orf19.4283*), a gene of unknown function. Using growth assays we showed that overexpression of *IPF2973* and *TRX1* influence growth in the presence of histatin 5. Further, we showed that both overexpression and deletion of *CaGPR1* (*orf19.1944*, *IPF2973* shows 29% homology with *S. cerevisiae GPR1*) also influences growth in the presence of histatin 5. Using microarray expression profiling of the early transcriptional responses to histatin 5 treatment, we identified 60 genes that are transcriptionally regulated by at least two-fold. Fifty of these genes were downregulated and ten were upregulated. Most downregulated genes are involved in carbohydrate, amino acid, and lipid-fatty acid metabolism, indicating that histatin 5 causes the cells to shut down their metabolism. Most of the upregulated genes (6 out of 10) had an unknown function, two were stress-responsive genes, one was involved in the lipid-fatty acid metabolism, and one was a Calcium-transporting ATPase. Eleven of the responsive genes were chosen for validation by RT Q-PCR, and all of them were confirmed to be differentially regulated. We also showed that overexpression of five of these genes (*POR1* (*orf19.1042*), *DDR48* (*orf19.4082*), *PGA3* (*orf19.2060*), *SNF31* (*orf19.7094*) and *CAT2* (*orf19.4591*)) has an influence on growth in the presence of histatin 5.

The histatin 5 peptide is internalized by *C. albicans* and targets to the mitochondria (Helmerhorst *et al.*, 1999), inhibiting respiration and inducing the formation of reactive oxygen species (ROS) (Helmerhorst *et al.*, 2001). These ROS can cause wide-ranging damage to macromolecules, resulting in physiological dysfunctions and eventually death. To protect against oxidative damage, cells can activate processes to maintain their redox balance (Wheeler & Grant, 2004). In this study we identified two genes (*TRX1* (*orf19.7611*) and *PGA3* (*orf19.2060*)) that form part of such a defense system, and whose overexpression makes *C. albicans* cells more resistant to histatin 5-induced cell death.

C. albicans contains two cytoplasmic thioredoxins, *TRX1* and *TRX2*. Thioredoxins are small heat-stable oxidoreductases containing two conserved cysteine residues in their active sites. They were identified as hydrogen donors for ribonucleotide reductase, but they are also required

for a number of metabolic enzymes that form a disulphide as part of their catalytic cycle. Thioredoxins are involved in many cellular processes, including repair of proteins damaged by oxidation. As in other species, yeast thioredoxins are antioxidants that play an important role in protection against ROS. *TRX2* seems to be the most important thioredoxin, but apparently it is required only during stationary phase. There is evidence for the functional redundancy of the thioredoxins as antioxidants in *S. cerevisiae*: overexpression of *TRX1* or *TRX2* leads to an increase in resistance to hydroperoxides. Both thioredoxins can act as cofactors for thioredoxin peroxidase-mediated resistance to hydroperoxides, and sensitivity of *trx1* and *trx2* mutants to hydroperoxides was not observed during exponential phase of growth (Garrido & Grant, 2002). *Candida TRX1* was picked up from the library screening as a gene whose overexpression increases resistance for histatin 5. The strain carrying the cDNA insert encoding *TRX1*, identified in the library screening, showed a clearly more resistant phenotype in the individual survival assay (clone M5, cell survival of 47% compared to 5% cell survival for the parental strain after a 2-h treatment with 100 µM histatin 5) (Fig. 2). Although we were able to show only minor overexpression in the constitutively *TRX1*-overexpressing strain (1.24 times higher *TRX1* mRNA level), the result of the growth assay clearly showed a more resistant phenotype, indicating the important role of thioredoxins in conferring partial resistance to histatin 5.

The second gene that was identified as involved in maintaining redox homeostasis is *PGA3* (*orf19.2060*). Pga3 shows homology with the *S. cerevisiae* Cu-Zn superoxide dismutase Sod1. Superoxide dismutases destroy superoxide radicals produced intracellularly by converting them into the less harmful hydrogen peroxide. *C. albicans* possesses three SOD enzymes that function in protecting the cell from oxidative stress: cytosolic Cu-Zn superoxide dismutase (*SOD1*) (Hwang *et al.*, 1999), mitochondrial Mn superoxide dismutase (*SOD2*) (Rhie *et al.*, 1999), and a cytosolic manganese-containing superoxide dismutase (*SOD3*) (Lamarre *et al.*, 2001). Three genes encoding products similar to known Cu-Zn-containing superoxide dismutases have also been identified: *PGA2*, *PGA3* and *PGA9*. Recently, the *PGA3* gene was characterized and renamed *SOD5*. The other two genes with similarity to known Cu-Zn-containing superoxide dismutases (*PGA2* and *PGA9*) were renamed *SOD4* and *SOD6*, respectively (Martchenko *et al.*, 2004). The Sod5 protein is involved in eliminating intracellular and extracellular superoxide radicals (Martchenko *et al.*, 2004), and is localized on the surface of *C. albicans* cells (Fradin *et al.*, 2005). The transcription of *SOD5* is upregulated during yeast to hyphal transition, during osmotic stress, upon treatment with hydrogen peroxide, and when cells are grown on nonfermentable substrates as sole carbon source (Martchenko *et al.*, 2004). All these are conditions in which the cells undergo high oxidative stress. When oxidative stress is induced in *C. albicans* using mild H_2O_2 conditions, *SOD1* and *SOD2* gene products are induced (Gonzalez-Parraga *et al.*, 2003). In *C.* albicans, disruption of *SOD1*, but not *SOD3*, had no effect on cell survival in medium containing H_2O_2 (Hwang *et al.*, 2002), indicating a

functional redundancy of superoxide dismutases. We showed that when *C. albicans* is treated with histatin 5, *PGA3* (*SOD5*) is differentially regulated by a factor of 4.2. Wunder *et al.* (2004) could not show effect on survival of cells from superoxide dismutase knockout strains of *S. cerevisiae* (*sod1/SOD2, SOD1/sod2* and *sod1/sod2*) and *C. albicans* (*sod1/sod1*) upon histatin 5 treatment (Wunder *et al.*, 2004). However, we could show that overexpression of *PGA3* (*SOD5*) in *C. albicans* increases its resistance to low concentrations of histatin 5. This discrepancy can be explained by the possible functional redundancy of the Sod proteins; it appears that Sod3 or Sod5, and possibly Sod4 and Sod6, can overcome mild H_2O_2 stress when *SOD1* is knocked out. In the *S. cerevisiae* double KO-strain (*sod1/sod2*) in which all known cytoplasmic superoxide dismutases are disrupted, no redundancy is possible (Wunder *et al.*, 2004). However, the *S. cerevisiae SOD* knockout strains were grown in glucose medium. *S. cerevisiae*, in contrast with *C. albicans*, is a Crabtree-positive yeast; when grown on a fermentable carbon source under aerobic conditions, the sugar is largely fermented to ethanol rather than respired. Grown aerobically on glucose, a fermentable carbon source, *S. cerevisiae* cells will largely perform fermentation. Consequently, the mitochondria are not active, they do not perform oxidative phosphorylation, and very little ROS is formed upon histatin 5 treatment. When grown on glucose, *S. cerevisiae* cells largely die upon histatin 5 treatment, probably not via the production of ROS, but via a mechanism probably not present in *C. albicans* (De Smet *et al.*, 2004). Therefore, disruption of superoxide dismutases will not affect survival of *S. cerevisiae* grown on glucose before treatment with histatin 5. When grown on a non-fermentable carbon source, it is more likely that the effect of disruption of the superoxide dismutases is seen.

In contrast to Wunder *et al.* (2004), we can conclude that ROS do play a role either directly or indirectly in histatin 5-induced cell death. Overexpression of *TRX1* or *PGA3* (*SOD5*), two genes involved in protection against oxidative stress, renders the cells more resistant to histatin 5. The protective effect of the overexpression of these proteins can be direct, by neutralizing the oxygen radicals (Fig. 10a), or indirect, by repairing proteins and lipids damaged by oxidation (Fig. 10b).

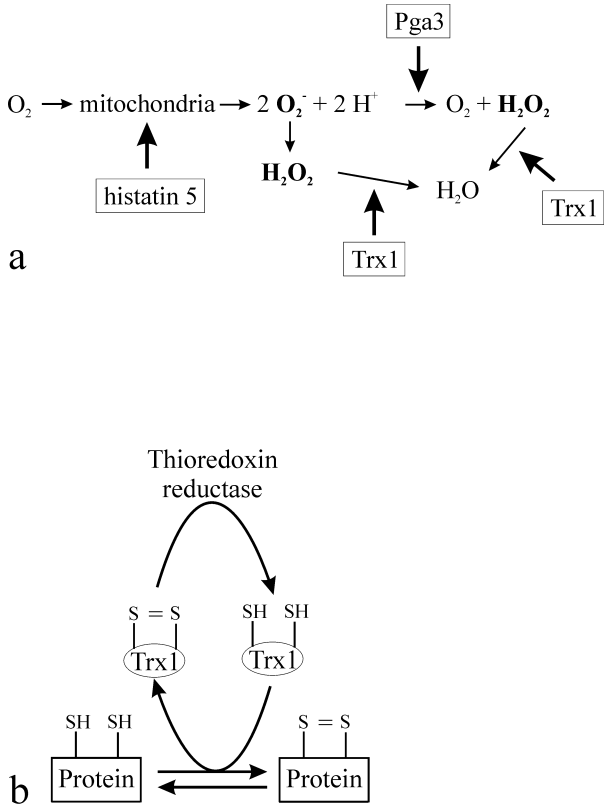

Figure 10: The protecting effect of the overexpression of *PGA3* or *TRX1*. (a) Direct protecting effect by neutralization of ROS. After internalization histatin 5 localizes to the mitochondria were it induces the production of ROS (in bold), e.g. the superoxide anion that is formed by the one-electron reduction of oxygen. In neutral aqueous solutions, the superoxide anion converts to hydrogen peroxide and molecular oxygen, a reaction that can be catalyzed by superoxide dismutases (e.g. Pga3). Hydrogen peroxide in turn can be converted to water by thioredoxin (Trx1). (b) Indirect protecting effect. Disulphide reduction of oxidatively damaged proteins by the thioredoxin system: a combined action of thioredoxin reductase and thioredoxin (Trx1).

One of the genes isolated from the library screening, *IPF2973* (*orf19.4283*), has an unknown function. Homology search revealed that the IPF2973 protein contains a Mov34 domain. The function of this domain is unclear, but it is found in the N-terminus of the proteasome regulatory subunits, eukaryotic initiation factor 3 (eIF3) subunits, and regulators of transcription factors. Further search revealed that it has 57% homology with a probable 26S proteasome regulatory subunit of *Schizosaccharomyces pombe*. Proteins containing a Mov34 domain could act as the regulatory subunit of the 26S proteasome, which is involved in the ATP-dependent degradation of ubiquitinated proteins. Overexpression of *IPF2973* in *C. albicans* CAI-4 renders the cells highly resistant to histatin 5. Although intracellular proteolytic degradation of histatins in *C. albicans* can occur, it is not generally used as a protective mechanism against histatin activity

(Ruissen *et al.*, 2003). By exposing *C. albicans* strain CA132A to increasing concentrations of histatin 3, histatin-resistant strains were generated. This resistance was not due to alterations in binding, internalization or degradation of histatin or efflux of ATP (Fitzgerald *et al.*, 2003). This indicates that protective mechanisms activated by exposure to histatin 3 may involve unidentified pathways downstream of binding and internalization. However, overexpression of *IPF2973* could lead to increased degradation of histatin 5 by the 26S proteasome, increasing survival. Another explanation could be increased degradation of damaged proteins by the 26S proteasome. Higher levels of the gene product of *IPF2973* could increase the efficiency of degradation. The 26S proteasome in yeast seems to be involved in resistance to various stresses: 26S proteasome mutants are hypersensitive to cadmium (Jungmann *et al.*, 1993) and methylmercury (Hwang *et al.*, 2002); the 26S proteasome is induced in heat stress and during late log-phase (Fischer *et al.*, 1994), and it is also involved in inhibition of cell death by degradation of the pro-apoptotic protein Smt1 (Ligr *et al.*, 2001). In *Mycobacterium tuberculosis*, the proteasome serves as a defense against oxidative or nitrosative stress (Darwin *et al.*, 2003). Many publications have reported on the relationship between protein oxidation and proteolysis in mammalian cells (Davies, 1986; Pacifici *et al.*, 1989; Grune *et al.*, 1995; Davies, 2001; Shringarpure *et al.*, 2001; Grune *et al.*, 2003), concluding that proteins are inherently susceptible to oxidative damage, which in turn alters proteolytic susceptibility. Overexpression of *IPF2973* makes the cells highly resistant to histatin 5-induced death, possibly by enhancing degradation of damaged proteins or internalized histatin 5.

Because of the weak nucleotide homology between *IPF2973* and *S. cerevisiae GPR1*, the *Candida* homologue of this G-protein coupled receptor was analyzed for involvement in histatin 5-induced cell death. A homologue of the *S. cerevisiae GPR1* was reported in *C. albicans* (Versele *et al.*, 2001) and its function was studied recently (Miwa *et al.*, 2004; Maidan *et al.*, 2005). Using growth assays of overexpression and knockout strains, we showed that overexpressing *Candida GPR1* (*orf19.1944*) increases resistance to histatin 5, whereas knocking it out increases sensitivity. The Gpr1 G-protein-coupled receptor was identified in *S. cerevisiae* as an upstream component in the cAMP-PKA pathway, and was shown to mediate activation of cAMP synthesis by glucose and sucrose. In *S. cerevisiae* cAMP signaling plays a central role in controlling metabolism, stress resistance and proliferation (Rolland *et al.*, 2002). In *C. albicans* Gpr1 regulates hyphal morphogenesis via the cAMP-PKA signaling cascade, and Gpa2, as in *S. cerevisiae*, acts through the cAMP-PKA pathway downstream of Gpr1 (Miwa *et al.*, 2004; Maidan *et al.*, 2005). When we analyzed the *GPA2* knockout in a growth assay, we observed a phenotype different from that of the *GPR1* knockout, indicating that Gpr1 and Gpa2 may function in overlapping but different pathways. How absence of Gpr1 enhances the sensitivity to histatin 5 remains to be established. It has been shown in *S. cerevisiae* that both Gpa2 and Gpr1 interact with Plc1 (Ansari *et al.*, 1999). Plc1 is a phosphatidylinositol (PI)-specific

phospholipase C that hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. The PI turnover pathway is well known in mammalian cells, and leads to activation of protein kinase C directly by diacylglycerol and by inositol triphosphate through intracellular Ca^{2+} release (Berridge, 1993). Furthermore, the Plc1 substrate PIP_2 is an important signal modulating the activity of membrane-bound proteins (Lee & Rhee, 1995). In yeast too a protein kinase C is involved in maintenance of cell integrity under hypotonic stress, heat shock, nutrient starvation, and during bud generation or cellular differentiation, through activation of the MAP kinase pathway (Heinisch *et al.*, 1999). Further, in *S. cerevisiae* Plc1 is involved in cytosolic transient glucose-induced calcium increase, which also requires the Gpr1/Gpa2 receptor/G-protein complex and glucose hexokinases. This increase in cytosolic Ca^{2+} concentration is mainly due to an influx from the external medium (Tisi *et al.*, 2002). Intracellular Ca^{2+} in yeast is a second messenger involved in several processes, including mating, nutrient sensing, stress response and cell cycle events. Overexpression of Gpr1 in *C. albicans* could lead to a more intense activation of Plc1, resulting in the strong activation of protein kinase C or a large increase in the intracellular Ca^{2+} concentration, which has an influence on susceptibility to histatin 5.

SNF31 (*orf19.7094*), a gene involved in glucose sensing, was found to be responsive to histatin 5. Upon histatin 5 treatment, *SNF31* was differentially regulated by a factor of 0.33. Ten-fold overexpression of *SNF31* results in increased growth rate in the presence of 5 µM histatin 5, but when the concentration is raised to 7.5 µM, the protective effect of this gene disappears. On the nucleotide level, Snf31 shows homology to *Ca*Snf3 (*orf19.5962*), a high-affinity glucose transporter. However, there are some important differences: Snf31 is shorter than Snf3 (527 AA and 749 AA, respectively), and lacks the functionally important C-terminal domain of Snf3; Snf31 has 12 transmembrane domains, while Snf3 has only 11. *SNF3* is not differentially regulated upon histatin 5 treatment of *C. albicans*. Recently is has also been shown that Snf31 but not Snf3 is strongly upregulated when *Candida* interacts with macrophages (Luongo *et al.*, 2005). Snf31 shows more structural homology with Hgt11 (*orf19.4527*) and Hgt12 (*orf19.3668*), two hexose transporters. These proteins have 12 transmembrane domains, are 546 AA long and have a short C-terminal domain. Furthermore, *HGT11* and *HGT12* are downregulated by a factor of 0.42 and 0.34, respectively, upon histatin 5 treatment. Besides Snf31, these are the only sugar transporters that are differentially regulated upon histatin 5 treatment. Recently, a large family of glucose transporter genes (*HGT1* to *HGT20*) was identified by screening genomic sequences, reverse-transcription PCR assays and phylogenetic analyses (Fan *et al.*, 2002). The transcript levels of *HGT11* were differentially regulated in the presence of drugs, e.g. cycloheximide, chloramphenicol and benomyl (Varma *et al.*, 2000). However, contrary to the downregulation of *HGT11* by histatin 5 treatment, its transcription was enhanced in the presence of these drugs. Furthermore, it was shown that transcription of

HGT11 and *HGT12* is upregulated in a *C. albicans* strain resistant to amphotericin B and fluconazol (Barker *et al.*, 2004). These results suggest that *HGT11* and *HGT12* could be linked to drug resistance in *C. albicans* (Varma *et al.*, 2000). The downregulation of *SNF31*, *HGT11* and *HGT12* upon histatin 5 treatment indicates that sugar transporters/sensors have an important role in the histatin 5-induced cell death process.

POR1 (*orf19.1042*) and *CAT2* (*orf19.4591*), two genes involved in transport of metabolites into the mitochondria, were also differentially regulated upon histatin 5 treatment; they were downregulated by a factor of 0.44 and 0.42, respectively. Strains overexpressing *POR1* or *CAT2* were more sensitive to histatin 5.

Compounds are transported across the outer mitochondrial membrane by mitochondrial porin (*POR1*), also known as the voltage-dependent anion-selective channel (VDAC). Death of *C. albicans* induced by histatin 5 requires coupled mitochondria performing oxidative phosphorylation. Knocking out *POR1* restricts the access of external NADH to the respiratory chain (Lee *et al.*, 1998). Because VDAC is the major pathway for NADH flux through the outer membrane of mitochondria, oxidative phosphorylation is hampered by *POR1* knockout; deletion strains grow slowly in the presence of a non-fermentable carbon source (Dihanich *et al.*, 1987). Overexpression of *POR1* in *S. cerevisiae* stimulates oxidative phosphorylation and increases respiration rate. Because the rate of cell death upon histatin 5 treatment depends on oxidative phosphorylation, strains overexpressing *POR1* are more sensitive to histatin 5.

Carnitine acetyltransferases belongs to a group of enzymes catalyzing the reversible acylation of L-carnitine. Carnitine acetyltransferase (*CAT2*) is specific for short chain fatty acids and has the highest activity on the acetyl moiety (Fritz *et al.*, 1963). In contrast to degradation of fatty acids (beta-oxidation) in mammals, their degradation in yeast takes place exclusively in peroxisomes (Kunau *et al.*, 1995). The acetyl-CoA produced is transported from the peroxisomes via the cytoplasm to the mitochondrial matrix for complete oxidation to CO_2 and H_2O. Two pathways for the transport of acetyl-CoA have been proposed (van Roermund *et al.*, 1995). The first involves peroxisomal conversion of acetyl-CoA into glyoxylate cycle intermediates, which are transported to the mitochondria. The second pathway involves peroxisomal conversion of acetyl-CoA into acetylcarnitine by *CAT2*, and its subsequent transport to the mitochondria. The acetyl-CoA is oxidized in the mitochondria via the Krebs cycle, with the formation of NADH, which is used in the respiratory chain. Overexpression of *CAT2* could increase the transport of acetyl-CoA to the mitochondria and thereby the rate of respiration. Again, enhancement of oxidative phosphorylation will render the cells more sensitive to histatin 5. It is clear that *C. albicans* cells treated with histatin 5 reduce their rates of respiration (downregulation of *POR1* and *CAT2*) and ATP-synthesis (downregulation of *ATP1* by a factor of 0.5), emphasizing the major role of the mitochondria in the histatin 5-induced cell death process.

The last gene whose overexpression influences cell growth in the presence of histatin 5 is *DDR48* (*orf19.4082*). *CaDDR48* was named for its homology to the common stress-responsive gene of *S. cerevisiae DDR48* (DNA damage responsive). In yeast, *DDR48* transcription increases in response to heat shock, osmotic stress, oxidative stress caused by cadmium, and treatments that produce DNA-lesions (McClanahan & McEntee, 1986; Miralles & Serrano, 1995; Momose & Iwahashi, 2001). Ddr48 is also implicated in the production or recovery of mutations (Treger & McEntee, 1990). In *C. albicans DDR48* is upregulated by treatment with itraconazole (De Backer *et al.*, 2001) and amphotericin B (Barker *et al.*, 2004), in a fluconazole-resistant *C. albicans* strain (Karababa *et al.*, 2004), and in cells undergoing the yeast-to-hyphal transition (Nantel *et al.*, 2002). Although Ddr48 is a stress protein and is upregulated upon histatin 5 treatment, its overexpression does not induce a more resistant phenotype.

Here we report the transcriptional response of *C. albicans* to histatin 5 treatment. We identified genes involved in the histatin 5-induced death of *C. albicans*, by phenotypically analyzing strains overexpressing responsive genes identified using transcriptional profiling and screening of a *C. albicans* overexpression library. Sixty genes were found to be differentially regulated upon histatin 5 treatment. Fifty of these genes were downregulated and ten were upregulated. Most downregulated genes are involved in carbohydrate, amino acid, and lipid-fatty acid metabolism, indicating that the cells shut down their metabolism upon incubation with histatin 5. Growth assays of 8 overexpression strains showed that they are either more resistant or more sensitive to histatin 5 treatment than the wild type strain. The functions whose overexpression renders the cells more resistant to histatin 5 are involved in oxidative stress response (*TRX1* and *PGA3*), in sensing extracellular nutrients (*GPR1* and *SNF31*), or have an unknown function (*IPF2973*). Those that make the cells more sensitive to histatin 5 are involved in transport of molecules (NADH, acetyl-CoA) into the mitochondria (*POR1* and *CAT2*) or in stress-response (*DDR48*). Interesting targets for the development of novel antifungal drugs are those functions whose deletion makes the cells more sensitive to external stress (e.g. incubation with histatin 5). We were able to identify one such function, Gpr1. Further research has to clarify whether Gpr1 can be used as a molecular target for the development of novel antifungal drugs. Other possible candidates are *TRX1*, *IPF2973*, *PGA3* and *SNF31*, whose expression makes cells more resistant to histatin 5. However, knockout studies have to be done to determine whether a more sensitive phenotype can be obtained.

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Deel IV
SAMENVATTING EN BESLUIT

SAMENVATTING EN BESLUIT

De laatste twee decennia is er een sterke stijging van het aantal opportunistische fungale infecties, ook de mortaliteit als gevolg van deze infecties is gestegen. Het organisme dat verantwoordelijk is voor de meeste van deze fungale infecties is *Candida albicans*. Een groot aantal antifungale middelen zijn gekend, maar deze zijn echter beperkt bruikbaar als therapeuticum wegens hun toxische bijwerkingen. Slechts enkele antifungale therapeutica zijn momenteel voorhanden en slechts sporadisch komen er nieuwe bij. Bovendien treedt er resistentie op tegen de frequent gebruikte antifungale middelen. Daarom zijn er nieuwe antifungale middelen nodig die inwerken op nieuwe moleculaire doelwitten en werken volgens een verschillend mechanisme in vergelijking met de gangbare antifungale middelen.

Het doel van dit doctoraatswerk was het induceren van celdood in *C. albicans* om daarna na te gaan welke genen betrokken zijn bij dit proces. De genen die betrokken zijn bij het celdoodproces kunnen gebruikt worden als doelwit voor de ontwikkeling van nieuwe antifungale middelen.

In het eerste hoofdstuk van de resultaten wordt beschreven hoe door de conditionele heterologe expressie van het pro-apoptotische eiwit Bax een celdoodproces geïnitieerd wordt in *C. albicans*. Door het afwijkend codongebruik van *C. albicans* bleek het onmogelijk om expressie te verkrijgen van het wild-type *BAX* gen van muis-oorsprong. Een synthetische, codon-geoptimaliseerde versie van dit gen resulteerde wel in expressie en veroorzaakte groei-inhibitie en celdood. Door de expressie van een fusie-eiwit van Bax met Gfp kon het celdood inducerende effect versterkt worden. We toonden aan dat dit versterkend effect te wijten is aan een verminderde proteolytische degradatie van het toxische Bax. Verder konden we, door middel van dit fusie-eiwit aantonen dat in *C. albicans* Bax met de mitochondriën colokaliseert en dat de mitochondriën, als gevolg van Bax-expressie, zich groeperen in een gebied nabij de kern. Dit was de eerste maal dat mitochondriale *clustering* in verband met celdood, beschreven werd in gist.

In het tweede hoofdstuk van de resultaten wordt de synthese en karakterisatie van het antifungale peptide histatine 5 beschreven. Dit synthetische peptide bleek dezelfde secundaire structuur te bezitten als het natuurlijke peptide en bleek in staat om celdood te induceren in *C. albicans* en *S. cerevisiae*. Vergeleken met *Candida* was *Saccharomyces* echter veel resistenter voor histatine 5-geïnduceerde celdood.
Twee belangrijke voordelen zijn aan histatine 5-geïnduceerde celdood verbonden vergeleken met Bax-geïnduceerde celdood. 1) Het is een natuurlijk voorkomend celdoodproces. Histatine 5

komt voor in het speeksel bij hogere primaten waar het ondermeer instaat voor het verhinderen van bacteriële en fungale infecties in de mondholte. De conditionele heterologe expressie van Bax in *Candida*, daarentegen, zorgt voor een artificieel celdoodproces, aangezien Bax niet voorkomt in gistcellen. 2) Histatine 5 wordt extracellulair aan de cellen toegevoegd. Dit heeft als voordeel dat de binding van het peptide aan de cel (al dan niet aan een receptor), internalisatie van het peptide en eventuele signalisatie naar latere evenementen in het celdoodproces ook onderzocht kunnen worden.

In een volgende hoofdstuk wordt een verklaring gegeven voor het verschil in gevoeligheid van *C. albicans* en *S. cerevisiae* voor histatine 5. De gevoeligheid van *S. cerevisiae* en *C. albicans* voor histatine 5 werd bepaald na een voorgroei van de cellen in fermenteerbare en niet-fermenteerbare koolstofbronnen en in de aan- of afwezigheid van een ontkoppelaar van de oxidatieve fosforylatie. *S. cerevisiae* bleek resistenter voor histatine 5 indien de cellen gegroeid werden in een fermenteerbare koostofbron dan wanneer groei gebeurde in een niet-fermenteerbare koolstofbron. Dit wijst op een belangrijke rol voor de oxidatieve fosforylatie in het histatine 5-geïnduceerde celdoodproces. Oxidatieve fosforylatie is noodzakelijk voor histatine 5-geïnduceerde celdood in *Candida albicans*. Dit bleek echter niet het geval te zijn voor *S. cerevisiae*, aangezien incubatie van CCCP-behandelde *S. cerevisiae* cellen met histatine 5 nog altijd in celdood resulteerde. Deze resultaten suggereren dat histatine 5-geïnduceerde celdood in *S. cerevisiae* verschilt van histatine 5-geïnduceerde celdood in *C. albicans*, met als gevolg dat het modelorganisme voor het onderzoek naar *C. albicans*, *S. cerevisiae*, niet kan gebruikt worden voor het onderzoek naar histatine 5-geïnduceerde celdood in *C. albicans*.

In het laatste hoofdstuk van de resultaten wordt beschreven hoe we trachtten genen te identificeren die betrokken zijn bij het histatine 5-geïnduceerde celdoodproces in *C. albicans*. Identificatie van functies die bij dit celdoodproces betrokken zijn, kan bijdragen om het moleculair werkingsmechanisme van histatine 5 beter te begrijpen. Bovendien kunnen deze functies gebruikt worden als doelwit voor de ontwikkeling van nieuwe antifungale drugs. De identificatie van deze functies werd ondernomen aan de hand van een screening van een overexpressie bibliotheek en aan de hand van microarray analyse. Een *C. albicans* cDNA bibliotheek werd achter de induceerbare *MAL2* promotor van *C. albicans* gecloneerd in een nieuw geconstrueerde replicatieve vector. *C. albicans* cellen, getransformeerd met deze overexpressie bibliotheek, werden vervolgens behandeld met histatine 5 en de cDNA fragmenten van de overlevende cellen werden gesequeneerd. Twee fragmenten kwamen overeen met een volledig gen: *TRX1*, een thioredoxine en *IPF2973*, een gen met onbekende functie. Aan de hand van groeitesten konden we aantonen dat overexpressie van *IPF2973* en *TRX1* groei beïnvloeden in de aanwezigheid van histatine 5. Verder toonden we aan dat zowel

overexpressie en deletie van *CaGPR1* (*IPF2973* vertoont 29% homologie met *GPR1* van S. cerevisiae) groei beïnvloedt in de aanwezigheid van histatine 5. Aan de hand van microarray analyse van de vroege transcriptionele respons van *C. albicans* op een histatine 5 behandeling, konden we 60 genen identificeren die een verandering in expressieniveau met tenminste een factor 2 vertoonden, na incubatie van de cellen met histatine 5. Vijftig van deze genen waren opgereguleerd, tien waren neergereguleerd. De meeste neergereguleerde genen, zijn genen die een rol hebben in het carbohydraat, aminozuur en lipide-vetzuur metabolisme, wat erop wijst dat histatine 5 ervoor zorgt dat de cellen hun metabolisme stilleggen. De meeste van de opgereguleerde genen (6 van de 10) hebben een onbekende functie, twee genen zijn betrokken bij de stress-respons, één gen is een calcium-transporterend ATPase en één gen is betrokken bij het lipide-vetzuur metabolisme. Uit deze 60 functies werden er 11 gekozen om de resultaten van de microarray analyse te bevestigen aan de hand van RT Q-PCR. Verder toonden we aan dat overexpressie van vijf van deze genen (*POR1, DDR48, PGA3, SNF31* and *CAT2*) een invloed hebben op de groei van *C. albicans* in de aanwezigheid van histatine 5.

Van de functies die we konden identificeren aan de hand van de screening van de cDNA bibliotheek en aan de hand van microarray analyse zijn er vier betrokken bij mitochondriale respiratie of oxidatieve stress (*POR1*, *CAT2*, *PGA3* en *TRX1*), twee hebben een functie in *nutrient sensing* (*GPR1* en *SNF31*), één heeft een onbekende functie (*IPF2973*), en een ander is betrokken bij stress (*DDR48*). Aangezien vier van de geïsoleerde functies (50% van de geteste functies) een rol spelen bij mitochondriale respiratie of oxidatieve stress (*POR1*, *CAT2*, *PGA3* en *TRX1*) (deel III, hoofdstuk 4) en we aangetoond hebben dat oxidatieve fosforylatie belangrijk en zelfs noodzakelijk is voor histatine 5-geïnduceerde celdood in, respectievelijk *S. cerevisiae* en *C. albicans* (deel III, hoofdstuk 2), kunnen we besluiten dat deze cellulaire processen belangrijk zijn voor histatine 5-geïnduceerded celdood. Ook genen die een functie hebben in *nutrient sensing* (*GPR1* en *SNF31*) kunnen blijkbaar een belangrijke rol spelen in het histatine 5-geïnduceerde celdoodproces. Verder onderzoek naar de rol van deze cellulaire processen in het histatine 5-geïnduceerde celdoodproces kan eventueel leiden tot de identificatie van meerdere genen die betrokken zijn bij dit celdoodproces.

De functies die de cel gevoeliger maken voor histatine 5 (indien ze overgeëxpresseerd worden) zijn betrokken bij het transport van moleculen (NADH, acetyl-CoA) in de mitochondriën (*POR1* en *CAT2*) of zijn betrokken bij de stress-respons (*DDR48*). De functies die interessante doelwitten zijn voor de ontwikkeling van nieuwe antifungale drugs zijn functies die, indien ze gedeleteerd worden, zorgen voor een meer gevoeliger fenotype voor externe stress (zoals incubatie met histatine 5). We zijn er in geslaagd om één zo'n functie te identificeren, Gpr1. Verder onderzoek moet ons leren of Gpr1 kan gebruikt worden als moleculair doelwit voor de

ontwikkeling van nieuwe antifungale drugs. Andere mogelijke kandidaten zijn *TRX1*, *IPF2973*, *PGA3* en *SNF31*, aangezien overexpressie van deze genen de cellen resistenter maakt voor histatine 5. Deletiestudies moeten echter nog uitgevoerd worden om te bepalen of deletiestammen van deze functies een gevoeliger fenotype vertonen.

Dit doctoraatswerk heeft uiteindelijk geleid tot enkele kandidaten die gebruikt kunnen worden als doelwit voor de ontwikkeling van nieuwe antifungale drugs. Verder hebben we het Bax-geïnduceerde celdoodproces in *C. albicans* en het histatine 5-geïnduceerde celdoodproces in *C. albicans* en *S. cerevisiae* meer in detail onderzocht. Ik hoop dat dit werk een startpunt kan zijn voor andere projecten in de zoektocht naar nieuwe antifungale middelen.

Deel V
ADDENDUM

ELSEVIER

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Old yellow enzyme interferes with Bax-induced NADPH loss and lipid peroxidation in yeast

Rieka Reekmans [a], Kris De Smet [a], Cuiying Chen [a], Paul Van Hummelen [b], Roland Contreras [a,*]

[a] *Unit of Fundamental and Applied Molecular Biology, Department for Molecular Biomedical Research, VIB-Ghent University, Technologiepark 927, B-9052 Zwijnaarde, Belgium*

[b] *Microarray Facility, Flanders Interuniversity Institute for Biotechnology (VIB), B-3000, Leuven, Belgium*

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Abstract

The yeast transcriptional response to murine Bax expression was compared with the changes induced by H_2O_2 treatment via microarray technology. Although most of the Bax-responsive genes were also triggered by H_2O_2 treatment, *OYE3*, *ICY2*, *MLS1* and *BTN2* were validated to have a Bax-specific transcriptional response not shared with the oxidative stress trigger. In knockout experiments, only deletion of *OYE3*, coding for yeast Old yellow enzyme, attenuated the rate of Bax-induced growth arrest, cell death and NADPH decrease. Lipid peroxidation was completely absent in Δ*OYE3* expressing Bax. However, the absence of *OYE3* sensitized yeast cells to H_2O_2-induced cell death, and increased the rate of NADPH decrease and lipid peroxidation. Our results clearly indicate that *OYE3* interferes with Bax- and H_2O_2-induced lipid peroxidation and cell death in *Saccharomyces cerevisiae*.

Keywords: Old yellow enzyme; Bax; Cell death; Oxidative stress; NADPH decrease; Lipid peroxidation

1. Introduction

Apoptosis is an evolutionarily conserved cell death process that eliminates unwanted cells that are damaged, aged, infected or useless, and plays important roles in embryonic development and in maintenance of tissue homeostasis [1]. Apoptosis is characterized by a number of morphological changes, including cell rounding and shrinkage, chromatin condensation, oligonucleosomal DNA cleavage, plasma membrane ruffling, and packaging of the contents of a dying cell into small membrane-bound vesicles [2,3]. Another typical feature of apoptosis is the exposure of phosphatidylserine on the outer leaflet of the plasma membrane [4]. Many of the typical biochemical and morphological changes in apoptotic cells are mediated by the proteolytic action of cysteine-dependent aspartate-specific proteases, designated caspases [5,6].

In yeast, apoptosis-like cell death is observed (i) in cells with a defective cell cycle [7], (ii) upon inactivation of Asf1 [8], Cdc13 [9], or Stm1 [10], (iii) in aged cells [11–13], (iv) after treatment with H_2O_2 [14], acetic acid [15], high salt [16], osmotin [17], α-factor [18], UV irradiation [19], and synthetic compounds [20–22], as well as (v) when there is nutrient imbalance [23]. Furthermore, the caspase-like protein Yca1 and the nuclear serine protease Nma111 are involved in yeast apoptosis-like cell death [24,25].

Members of the Bcl-2 (B-cell lymphoma) family of proteins are important regulators of apoptosis. This family is composed of anti-apoptotic members (such as

* Corresponding author. Tel.: +32 9 331 3631; fax: +32 9 331 3502/3609.
E-mail address: roland.contreras@dmbr.ugent.be (R. Contreras).

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Bcl-2 and Bxl-X_L), pro-apoptotic members (such as Bax and Bak), and a large group of BH3-only death proteins that trigger apoptosis or sensitize for it. The ratio between anti- and pro-apoptotic proteins determines the susceptibility of a particular cell to apoptosis [26–28]. Hyperexpression of the pro-apoptotic protein Bax is sufficient to induce apoptosis in mammalian cells [29]. In addition, ectopic overexpression of Bax is toxic to *Saccharomyces cerevisiae* [30,31], inducing a variety of morphological abnormalities, including loose chromatin condensation, mitochondrial swelling, DNA strand breaks and plasma membrane blebbing [31,32]. However, there is no evidence for compact chromatin condensation or oligonucleosomal DNA degradation [31], probably due to the absence of linker DNA between yeast nucleosomes [33]. Bax toxicity is also observed in other organisms [34–38].

In mammalian and *S. cerevisiae* cells, Bax is targeted to mitochondria [31,39], inserts into their membranes [40,41], triggers cytochrome *c* release [42,43], and induces the production of reactive oxygen species [14,29]. Its expression induces mitochondrial damage [44,45], and leads to mitochondrial dysfunction [43,46], probably due to the formation of a channel in the outer mitochondrial membrane [47] by direct pore formation [48,49]. The precise mechanism of mitochondrial permeabilisation remains unclear [50] and different molecular mechanisms have been proposed [51]. In mammalian cells, Bax-mediated alterations in mitochondrial and endoplasmic reticulum Ca^{2+} levels serve as upstream signals for cytochrome *c* release [52–55].

Several yeast mutants impaired in Bax-induced yeast cell death have been selected [46,56–62] and several suppressors of Bax-induced yeast cell death have been discovered [63–70]. Together, these mutants and suppressors implicate mitochondrial physiology, oxidative stress, vesicular transport and autophagy in determining the final effect of Bax toxicity.

To focus on the mechanism by which oxidative stress is elicited following Bax protein expression, one has to theoretically subtract from the cellular effects of Bax those effects that are caused by the oxidative stress associated with its expression. In the present study, the transcriptional response to Bax expression in *S. cerevisiae* was compared with that elicited by an equally toxic H_2O_2 treatment, using microarray technology.

2. Materials and methods

2.1. Cloning of the mouse Bax coding sequence

cDNA encoding the mouse Bax-α protein (EMBL L22472) was amplified from an EL4/13.18 mouse thymoma cDNA library (BCCM™/LMBP-LIB15) by PCR (Polymerase Chain Reaction) using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the primers 5′-ATGGACGGGTCCGGGAGCAG-3′ and 5′-TCAGCCCATCTTC-TTCCAGATGGTGAG-3′. The resultant PCR product was cloned using standard procedures in a *Hinc*II-opened pUC19 plasmid [71], to produce pUC19B.

2.2. Plasmid constructions

The 2-mu ori and the *URA3* marker gene were consecutively excised from pUT332 [72] by digestion followed by self-ligation using *Cla*I and *Bgl*II, respectively, to produce YIpUT332. A *Bam*HI–*Hin*dIII fragment containing the *GAL1* promoter was then ligated into the *Bgl*II–*Hin*dIII-opened YIpUT332 plasmid, thereby obtaining YIpUTGAL1p. This plasmid was linearized with *Hin*dIII, end-blunted, digested with *Xba*I, and ligated to an *Fsp*I–*Xba*I fragment containing the *FLP1* terminator, creating plasmid YIpUT. Insertion of a Ty δ element as a blunted-ended *Eco*RI–*Bsa*AI fragment in the *Kpn*I–*Aat*II-opened and blunted YIpUT resulted in YIpUTy. Subsequent insertion of the *LEU2* marker gene, as a blunted *Bsa*AI–*Bsr*GI fragment, in the *Bam*HI-opened and blunted YIpUTy created plasmid YIpUTyL (pSCTyGAL1-L) (LMBP 4913). Mouse *bax* cDNA was excised from pUC19B by digestion with *Xba*I and *Hin*dIII and subcloned into the *Xba*I–*Hin*dIII-opened plasmids pSCTyGAL1-L and p415GALL [73] (LMBP 4032), obtaining pSCTyGAL1mBAX-L (LMBP 3871) as an integrative expression plasmid for *GAL1*-driven Bax expression, and p415GALLmBAX (LMBP 4575) as a centromeric plasmid for *GALL*-driven Bax expression. Plasmids indicated with an LMBP number were deposited at BCCM (http://www.dmbr.Ugent.be/lmbp).

2.3. Yeast strains and growth conditions

The *S. cerevisiae* strains used were INVSc1 (*MAT*α *his*3Δ1 *leu*2-3, 112 *trp*1-289 *ura*3-52) (Invitrogen, Gaithersburg, MD), BY4742 (*MAT*α *his*3Δ1 *leu*2Δ0 *lys*2Δ0 *ura*3Δ0), and their knockout derivatives (Euroscarf, Frankfurt, Germany). Yeast strains were grown at 30 °C and 250 rpm in rich YPD-medium (2% glucose, 2% Bacto-peptone, 1% yeast extract) or SD-medium (2% glucose, 0.67% yeast nitrogen base, 0.5% ammonium sulphate), either minimally supplemented (minimal SD-medium) with amino acids (0.2 mM leucine, 0.2 mM lysine, 0.1 mM histidine, 0.1 mM tryptophan) and 0.2 mM uracil, or fully supplemented (synthetic SD-medium) with a complete amino acid mix containing uracil. For transformant selection, leucine was dropped out (selective SD-medium) where appropriate. SG-medium, used for the induction of the galactose regulated promoters, differs from SD-medium only by the replacement of glucose with 2% galactose as carbon

source. To prepare plates, media were solidified with 2% agar. The *S. cerevisiae* strains were transformed by the lithium acetate method [74], and selected on leucine-deficient SD-plates. The pSCTyGAL1-L and pSCTyGAL1mBAX-L plasmids were linearized at the Ty δ element with *Xho*I prior to transformation to ensure targeted integration. INVSc1 and BY4742 strains were used for integrative and episomal transformation, respectively.

2.4. RNA preparation following Bax expression or H_2O_2 treatment

2.4.1. Induction of Bax expression – precultures of strains

INVSc1::pSCTyGAL1mBAX-L and INVSc1::pSCTyGAL1-L were grown overnight in selective minimal SD-medium. They were diluted at least 500-fold in the same growth medium and grown to an OD_{600} of 1.0. Subsequently, the cells were washed three times with water, resuspended in 100 ml selective minimal SG-medium, and incubated for 1 h.

2.4.2. Challenge with H_2O_2

The yeast strain INVSc1::pSCTyGAL1-L was grown as described above, but the selective minimal SG-medium was supplemented with different concentrations of H_2O_2 ranging from 0.1 to 0.5 mM.

2.4.3. Total RNA extraction

After a 1 h of Bax protein induction, H_2O_2-treatment, or mock-treatment, about 10^9 cells were harvested by centrifugation at 2200*g* for 5 min at 4 °C, and washed with ice-cold sterile water. Cells were then combined with 1 ml RNApure™ reagent (Genhunter Corporation, Nashville, NY) and 1 g of glass beads (0.5 mm diameter), and broken by thorough mixing. The lysate was combined with 150 μl chloroform and centrifuged at 20,000*g* for 10 min at 4 °C. The RNA in the supernatant was precipitated with an equal volume of isopropanol for 10 min on ice, pelleted at 20,000*g* for 10 min at 4 °C, and washed with 70% ice-cold ethanol. The RNA was resuspended in 50 μl RNAse-free water. Typically, about 1 mg total RNA could be extracted from 10^9 cells. The purity and quality of the RNA preparations were checked by spectrophotometry (A_{260}/A_{280} and A_{260}/A_{230}) and by an RNA assay using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany).

2.5. Gene expression profiling using microarray technology

2.5.1. Construction of the microarrays

The yeast microarrays consisted of 70-mer oligos (Qiagen, Hilden, Germany) spotted on Type VII silane-coated slides, and represented 6307 well-characterized yeast genes with high specificity. The oligos were designed for optimal discriminatory potential between homologous genes. The microarrays were kindly provided by the Microarray Department of the Swammerdam Institute for Life Sciences at the University of Amsterdam.

2.5.2. RNA amplification and labeling

RNA was amplified in duplicate using a modified version of the in vitro transcription procedure [75]. Briefly, 5 μg total RNA was converted to double stranded cDNA using 5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGT$_{24}$(ACG)-3′ (Eurogentec, Seraing, Belgium) as anchored oligo-dT containing the T7 promoter. RNA was transcribed from this cDNA using T7-in vitro transcriptase to produce 10–30 μg amplified RNA. From the latter, 1 μg was labeled with the CyScribe Direct™ mRNA labeling kit (Amersham Biosciences, Piscataway, NJ) in either Cy3 or Cy5 (Amersham Biosciences), and purified using the CyScribe™ GFX™ purification kit (Amersham Biosciences). The resultant probes were analyzed for amplification yield and incorporation efficiency by measuring the RNA concentration at 280 nm, Cy3 incorporation at 550 nm, and Cy5 incorporation at 650 nm using a Nanodrop spectrophotometer (NanoDrop Technologies, Rockland, DE). Typically, a labeling density of 44 pmol CyDye μg^{-1} was obtained, corresponding to an incorporation rate of one fluorochrome each 70 nucleotides. For each probe, 40 pmol of incorporated Cy5 or Cy3 were added to 210 μl hybridization solution containing 50% formamide and 0.1% SDS in 1 × Hybridization Buffer (Amersham BioSciences).

2.5.3. Array hybridization and post-hybridization processes

Hybridization and post-hybridization washings were performed at 37 °C in an Automated Slide Processor (Amersham BioSciences, Piscataway, NJ). Post-hybridization washing was performed in 1 × SSC, 0.1% SDS, followed by 0.1 × SSC, 0.1% SDS, and finally 0.1 × SSC. Arrays were scanned at 532 and 635 nm using the Agilent DNA MicroArray scanner (Agilent Technologies, Waldbronn, Germany). Images were analyzed with ArrayVision™ (Imaging Research Inc, Ont., Canada), and spot intensities were measured as median intensities corrected for local background (sMedianDensity). Data were normalized for dye intensity differences using a Lowess-procedure [76] between ratio $\log_2$ (Cy5/Cy3) and average signal intensity $\log_2$(Cy5 × Cy3). Signals from non-yeast controls could not be distinguished from background. Subsequently, between-slide normalization was performed with the MARAN web application (http://www.esat.kuleuven.ac.be/maran) using a generic model for sequential

analysis of variance [77]. Regulated gene candidates were selected when expression level changes were at least 2-fold, the coefficient of variation between the ratios was <0.5, and the signal to background ratios were at least 10.

2.6. Subtractive gene expression profiling strategy

The normalized data of the Bax-responsive genes were compared with the normalized data obtained after H_2O_2 treatment in order to eliminate the common gene expression changes. The Bax-specific genes were defined as those Bax-responsive genes for which the expression level due to Bax was at least 2-fold more manifest or in the opposite sense compared with the expression difference due to H_2O_2.

2.7. Real-time quantitative polymerase chain reaction

Total RNA (5 μg of the same pool used in primary gene expression profiling) was incubated with 2 units of RQ1 RNAse-free DNAse (Promega, Madison, WI) at 37 °C for 30 min. The volume was adjusted to 200 μl with RNAse-free water and the mixture desalted using Microcon-YM100 (Millipore, Billerica, MA). The eluate, typically about 5 μl, was added to 2 μg oligo-dT, the volume adjusted to 10 μl with RNAse-free water, and the mixture incubated at 70 °C for 10 min. After cooling on ice, the following components were added: first-strand buffer (Life Technologies, Paisley, UK), 3.3 mM dithiothreitol, 40 units RNase Block (Stratagene, La Jolla, CA), 1 mM of each dNTP (Amersham Pharmacia Biotec, Uppsala, Sweden), and 200 units Superscript II reverse transcriptase (Life Technologies). The 25 μl mixture was incubated for 1 h at 42 °C. The resulting first-strand cDNA was used as a template for the subsequent real-time quantitative polymerase chain reaction (RT Q-PCR), which was performed in 1 × SYBR Green PCR buffer containing ROX as passive reference (Eurogentec, Seraing, Belgium), 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 300 nM primers and 0.025 units of Ampli*Taq* Gold® DNA polymerase. SYBR Green was used at a 1:66,000 dilution. RT Q-PCR amplification was performed in triplicate using the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The gradual increase in fluorescence due to the formation of a complex between SYBR Green and double-stranded DNA was monitored in real-time using an ABI prism 7700 (Perkin–Elmer Applied Biosystems, Foster City, CA). Primers were designed with the assistance of the PrimerExpress™ software (Perkin–Elmer Applied Biosystems). The following forward and reverse gene-specific primers were used: 5′-ACGTTTACGATATCAGGAAGGATTGT-3′ and 5′-AGCTTCTTTGACGTAGTCCTGGTT-3′ (*PRC1*); 5′-GAATGCTCCTGTGAACACCTATATGA-3′ and 5′-TGATCGCGTCGTAGAGATTAACTT-3′ (*MLS1*); 5′-GGCTGTTGGTTTGAGTGACTCTTT-3′ and 5′-CGACCATATCTCTCAACAACGTAAA-3′ (*HXT7*); 5′-AGACAGGAGTAAAAGAAGAAGGTTCAAA-3′ and 5′-CCGGTGTCAGACCAGGATATCTAT-3′ (*YIL127C*); 5′-GGTAAGCCAGGCGATGAGATT-3′ and 5′-TTGTTCATGCGATTGCAAATTT-3′ (*STF2*); 5′-AAACGAGGTGTCAGCAGTGTCATA-3′ and 5′-CTTTACCTGACCTGAATGTCTACCAA-3′ (*CYC7*); 5′-GAGTCAGAATGCACGCACACTAA-3′ and 5′-GCGGCGTTGCCGTTAA-3′ (*ICY2*); 5′-GGCTGCTCAAAAGTCTTTCAGAAT-3′ and 5′-TCCAGTTTCTTCTCTTAGCGTTGTAA-3′ (*RPL39*); 5′-CGCCGATGGTGTAGAAATTCAT-3′ and 5′-CGATCGTTCCGCCGTATT-3′ (*OYE3*); 5′-CCAGTGAGCTATTATCCAGAATGTAAA-3′ and 5′-GCATACCTCAAAGGTTCTGAACAA-3′ (*BTN2*); 5′-TGAAGCCGAAAATTGTGTTGTT-3′ and 5′-GTATATAGCTTCAAAAGCTTGGTAAATTTCT-3′ (*TBP1*); and 5′-AATATATCATCAGTGACAGGTGCCATT-3′ and 5′-AGCGCCTGTAGCGAAATCA-3′ (*UBC1*). *TBP1* and *UBC1* were used as reference genes for normalization. Specificity of the amplicons generated during the PCR reactions was confirmed by melting curve generation using Dissociation Curves 1.0 supplied by the manufacturer (Perkin–Elmer). Gene expression was quantified on the basis of the threshold cycle values (C_t) at which a statistically significant increase in ROX-normalized fluorescence intensity was first detected. The C_t-value (as the mean of three independent RT Q-PCR reactions) for the gene of interest (target) was normalized to both reference genes within each sample, and this normalized target value was compared between the samples of interest (Bax expression or H_2O_2 treatment) and the control sample (untreated), generating a $\Delta\Delta C_t$ value. The normalized level of target mRNA in the sample relative to the control was expressed as $2^{-\Delta\Delta C_t}$.

2.8. Growth assay

Precultures were grown overnight in selective synthetic SD-medium, diluted at least 500-fold in the same growth medium, and grown to early logarithmic phase. The cultures were washed three times and resuspended at 5×10^7 cells ml^{-1} in sterile water. A sample of 5 μl from each suspension (250,000 cells) was then inoculated in 195 μl selective synthetic SD- or SG-medium (Honeywell plate format). Cells were grown at 30 °C for 72 h with intermittent shaking for 10 s at 10 min intervals. Growth was assayed using a Bioscreen C (Labsystems, Helsinki, Finland), measuring optical densities every hour. The logistic growth curve was chosen for modeling purposes [78], and the growth rate of each culture was calculated based on the parameters of the fitted growth equation ($R^2 > 0.999$).

2.9. Clonogenic survival assay

Precultures were grown overnight in synthetic SD-medium (leucine drop-out in the case of transformants), diluted at least 500-fold in the same growth medium, and grown to early logarithmic phase. The cells were washed three times with sterile water if the carbon source had to be switched from glucose to galactose. For induction of Bax expression, cells were resuspended in selective synthetic SG-medium. After specific time intervals, 1000 colony-forming units, estimated from measurement of OD_{600}, were plated on synthetic semi-solid SD-medium (leucine-deficient in the case of transformants) using 4-mm diameter sterile glass beads. The plates were incubated for 3 days and colonies were counted automatically with the Scanalyzer (LemnaTec, Würselen, Germany). For each time point, percentage survival was calculated with reference to the mock treatment.

2.10. Immunological detection of Bax protein

About 10^8 cells were harvested by centrifugation at 2200*g* and washed with sterile water. They were broken with 0.5 g glass beads (0.5 mm) by vigorous vortexing for 5 min in 200 μl ice-cold lysis buffer (50 mM Tris–HCl, pH 8, 0.1% Triton X-100, 0.5% SDS) containing 1 × Complete™ protease inhibitor (Roche, Basel, Switzerland). The lysate was then centrifuged at 6000*g* for 5 min at 4 °C to remove cell debris and glass beads. Protein concentrations were determined with the Bicinchonic Acid Protein assay (Pierce, Rockford, IL). Samples (30 μg) were fractionated by SDS–PAGE (15% acrylamide) and transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG). Membranes were blocked overnight with 2% skimmed milk in PBS supplemented with 0.05% Tween-20, and probed with a mouse anti-Bax monoclonal antibody (B9, sc-7480) (Santa Cruz Biotechnology, Santa Cruz, CA), followed by horseradish-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO). The protein bands were visualized by chemiluminescence using Western Lightning™ (Perkin–Elmer Life Sciences, Boston, MA).

2.11. NADPH assay

BY4742 and Δ*OYE3* yeast cells transformed with p415GALLmBAX were grown in selective synthetic SD-medium. Samples were taken prior to the carbon switch (0 h) and at three additional time points (6, 12 and 24 h) after resuspension in selective synthetic SG-medium. To extract the pyridine nucleotides from the yeast, about 10^8 cells were harvested, washed three times with ice-cold water, and resuspended in 250 μl ice-cold extraction buffer (100 mM Tris–HCl, pH 8, 10 mM EDTA and 0.05% Triton X-100). Cells were broken with 0.5 g glass beads (0.5 mm) by vigorous vortexing for 5 min at 4 °C. The lysates were centrifuged at 6000*g* for 5 min at 4 °C, and the supernatants were analyzed immediately. NADPH was assayed spectrophotometrically (Shimadzu, Kyoto, Japan) as described [79] and NADPH concentrations were normalized to the protein contents of the samples.

2.12. Assays for oxidative protein and DNA damage

BY4742 p415GALLmBAX transformants were grown in selective synthetic SD-medium and reference samples were taken prior to the carbohydrate switch (0 h). Upon resuspension in selective synthetic SG-medium, cells were harvested at additional time points (12 and 24 h).

2.12.1. Assay for oxidative protein damage

Approximately 3×10^8 cells were harvested, washed with sterile water, and broken with 0.5 g glass beads (0.5 mm) in 200 μl ice-cold breaking buffer (15% glycerol, 2 mM EDTA) containing 1 × Complete™ protease inhibitor (Roche, Basel, Switzerland). Beads and cell debris were removed by centrifugation, and protein concentrations were determined. Oxidative protein damage was determined [80] by reaction with 2,4-dinitrophenylhydrazine (DNPH) [81] using the OxyBlot™ Oxidized Protein Detection Kit (Intergen, Purchase, NY). DNP-derivatized proteins were detected using a rabbit anti-DNP antibody and horseradish-conjugated goat anti-rabbit IgG. The protein dots were visualized by chemiluminescence using Western Lightning™ (Perkin–Elmer Life Sciences, Boston, MA), and the signals were analyzed with the LumiAnalysis™ image analysis software (Roche, Basel, Switzerland). The dot blots were then washed extensively, and the proteins were stained with Amido Black (0.1% in 45% methanol/10% acetic acid) for 15 min. After destaining with 45% methanol/10% acetic acid, the signal was analyzed using the Quantity One® program (BioRad, Richmond, CA), and the immunostaining signals were normalized to the total protein content of the dot.

2.12.2. Assay for oxidative mitochondrial DNA damage

Total DNA was prepared from 3×10^8 cells using MasterPure™ (Epicentre, Madison, WI) according to the guidelines provided by the manufacturer. DNA concentration was determined using the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR) and the CytoFluor fluorescence reader (PerSeptive Biosystems, Framingham, MA), employing λ DNA as standard. Oxidative mitochondrial DNA damage was measured by quantitative PCR, as the degree by which the amplification of a large (error-prone) amplicon (6.9 kb) was hampered relative to a short (error-resistant) amplicon (0.3 kb) of the mitochondrial *COX1* gene [82].

2.13. Assay for lipid peroxidation

BY4742 and Δ*OYE3* yeast cells transformed with p415GALLmBAX were grown in selective synthetic SD-medium. Samples were taken prior to the carbohydrate switch (0 h) and at two additional time points (12 and 24 h) after resuspension in selective synthetic SG-medium. BY4742 and Δ*OYE3* yeast cells were grown in synthetic SD-medium and treated with 0.5, 1.0 or 2.0 mM H_2O_2 for 30 min. The extent of lipid peroxidation was measured by thiobarbituric acid (TBA) reactivity with malondialdehyde (MDA). About 5×10^8 cells were washed with sterile water and resuspended in 125 μl 25% HCl. Next, 125 μl of 1% (w/v) TBA in 50-mM NaOH were added. Tubes were incubated at 85 °C for 30 min and cooled. The chromogen was extracted by the addition of 250 μl 1-butanol, vigorous vortexing, and centrifugation for 1 min at 6000*g*. The upper organic phase was used for fluorescence analysis (excitation: 530 nm, emission: 590 nm) using a CytoFluor fluorescence reader (PerSeptive Biosystems, Framingham, MA). To determine the MDA concentration in the samples, 1,1,3,3-tetramethoxypropane, an MDA precursor in mild acidity [83], was used as a standard at concentrations ranging from 2 to 200 μM.

3. Results

3.1. Achieving equal toxicity with Bax protein expression and H_2O_2 treatment

S. cerevisiae cells (INVSc1 strain) were transformed with the expression plasmid pSCTyGAL1mBAX-L or with the parental plasmid pSCTyGAL1-L. The Ty δ element present in both plasmids allowed stable single-copy integration in the INVSc1 genome, as confirmed by Southern analysis (data not shown).

Induction of Bax expression resulted in a time-dependent increase in the amount of dead cells, with 10% of the cells dying within 1 h of Bax induction. For comparison of Bax-induced with oxygen stress-induced cell death, we optimized the H_2O_2 dose so that, under the same growth conditions, a 1-h treatment would be as lethal as 1 h of Bax induction. Yeast cells were challenged with increasing concentrations of H_2O_2 and the percentage of cell death after 1 h was determined by survival assay. Incubation in 0.1 mM H_2O_2 for 1 h resulted in the same level of mortality observed after 1 h of Bax induction (data not shown).

3.2. Comparison of the transcriptional responses upon Bax expression and H_2O_2 treatment

Total RNA was extracted after a 1-h Bax-induction, a 1-h mock (control) or H_2O_2 treatment (0.1 mM), and used for microarray transcriptional profiling. The experiment had a triangular loop design comprising control (green) versus Bax (red), Bax (green) versus H_2O_2 (red), and H_2O_2 (green) versus control (red). Digital images of the fluorescent signals from the hybridized slides were normalized for dye intensity differences by Lowess fitting and for between-slide normalization using MARAN for variance analysis. Inspection of the signals revealed that 75 genes had mRNA steady-state levels that changed at least 2-fold after 1 h of Bax induction (Table 1).

Next, the changes in normalized spot intensity due to Bax expression and due to H_2O_2 treatment were compared, focusing on the 75 Bax-responsive genes. Out of these genes, we could detect nine genes for which the mRNA expression level changed to a higher degree with Bax expression than with H_2O_2 treatment (*PRC1*, *MLS1*, *HXT7*, *YIL127C*, *STF2*, *CYC7*, *ICY2*, *OYE3*, *BTN2*). We also detected one gene for which the transcriptional level was downregulated by Bax but upregulated by H_2O_2 (*RPL39*) (Table 2). These 10 genes were designated as Bax-specific transcriptional response candidates.

The level of expression of the 10 Bax-specific candidate genes was verified by a real-time PCR procedure relative to the genes *TBP1* and *UBC1*, for which the transcript levels remained stable upon Bax-expression or H_2O_2 addition (data not shown). The primers used for this validation are listed in Section 2. Four of the 10 candidate genes (*MLS1*, *OYE3*, *ICY2* and *BTN2*) were confirmed to have a Bax-specific transcriptional regulation (Table 2). We next wanted to determine whether knockout of these genes influenced Bax-induced cell death.

3.3. Bax-induced cell death in strains knocked out for genes specifically responsive to Bax

The plasmids p415GALL and p415GALLmBAX were transformed into BY4742, Δ*MLS1*, Δ*OYE3*, Δ*ICY2* and Δ*BTN2*, and their transformants were analyzed by a growth assay in selective synthetic SG-medium (Fig. 1). For the empty vector transformants, the growth of all four knockout strains did not differ from that of the control BY4742. For p415GALL-mBAX transformants, the growth rate of Δ*OYE3* was higher than that of BY4742, but none of the other three knockout strains demonstrated any difference. Besides visual inspection, a logistic growth curve [78] was used for modeling purposes. The growth rate was calculated as the first derivative at the inflection point, and the growth rate ratio of the Bax-expressing population to the control transformant was calculated for each strain (Fig. 1). The absence of *OYE3* resulted in a 19.5% (standard deviation: 4.0%) improvement in the growth rate ratio of the Bax-expressing population.

Table 1
Classification of the 75 Bax-responsive genes determined by microarray transcriptional profiling

ORF	Gene	Description	Fold change
Carbohydrate metabolism			
YNL117W	*MLS1*	Malate synthase (glyoxylate cycle)	+2.09
YBR218C	*PYC2*	Gluconeogenic pyruvate carboxylase, NADPH regeneration	−2.08
YMR169C	*ALD3*	Aldehyde dehydrogenase, induced by oxidative shock	−2.50
Cell polarity			
YKR055W	*RHO4*	Ras homolog involved in actin filament organisation	−2.70
Degradation			
YMR297W	*PRC1*	Carboxypeptidase Y (Proteinase C) for vacuolar protein catabolism	+2.29
YLL039C	*UBI4*	Ubiquitin, transcriptionally induced in stress conditions	+2.10
YBR208C	*DUR80*	Urea amidolyase, degrades urea to CO_2 and NH_3	−2.00
YBR058C	*UBP14*	Ubiquitin-specific protease, negative regulator of gluconeogenesis	−2.04
YLR351C	*NIT3*	Hydrolase acting on carbon-nitrogen (no peptide) bonds	−2.08
YGR020C	*VMA7*	Subunit f of vacuolar H^+-ATPase, for vacuolar acidification	−2.17
DNA synthesis			
YNL102W	*POL1*	DNA polymerase I α-subunit p180	+2.12
YMR234W	*RNH1*	Ribonuclease H1, involved in DNA replication	+2.12
YJR068W	*RFC2*	Replication factor C, involved in cell cycle checkpoint	+2.10
Energy generation			
YBR039W	*ATP3*	γ-Subunit of the F_1 sector of mitochondrial F_1F_0 ATP synthase	+2.18
YPL078C	*ATP4*	Subunit b of the stator stalk of mitochondrial F_1F_0 ATP synthase	+2.16
YGL256W	*ADH4*	Alcohol dehydrogenase type IV for glucose fermentation	+2.00
YGR008C	*STF2*	Stabilizing factor of the F_1F_0 ATP synthase	−2.04
YEL039C	*CYC7*	Cytochrome *c* isoform 2 involved in mitochondrial electron transport	−2.08
Intracellular transport			
YPL094C	*SEC62*	Membrane component of ER protein cotranslocation apparatus	+2.16
YBL102W	*SFT2*	Protein involved in Golgi-to-endosome transport	+2.06
YMR195W	*ICY1*	Protein involved in nuclear transport	−2.13
YPL250C	*ICY2*	Protein involved in nuclear transport	−2.17
YJR058C	*APS2*	Protein involved in vesicle-mediated transport	−2.17
YNL036W	*NCE103*	Endogenous substrate for nonclassical export	−2.27
YGR142W	*BTN2*	Protein involved in intracellular protein transport	−2.50
YER103W	*SSA4*	Membrane component of ER protein cotranslocation apparatus	−3.13
Lipid metabolism			
YGR157W	*CHO2*	Phosphatidyl-ethanolamine *N*-methyltransferase	−2.13
YPL171C	*OYE3*	NADPH dehydrogenase, possibly involved in sterol metabolism	−2.22
Protein modifications			
YER123W	*YCK3*	Protein involved in amino acid phosphorylation	+2.27
YKL035W	*UGP1*	UTP-glucose-1-phosphate uridylyltransferase for glycosylation	−2.13
YGR161C	*RTS3*	Protein involved in amino acid dephosphorylation	−2.78
Protein synthesis			
YOL077C	*BRX1*	Essential protein required for biogenesis of the 60S ribosomal subunit	+2.55
YOL040C	*RPS15*	Protein component of the 40S ribosomal subunit	−2.00
YJL189W	*RPL39*	Protein component of the 60S ribosomal subunit	−2.22
YER035W	*EDC2*	RNA-binding protein, activates mRNA decapping	−2.56
Small molecule transport			
YDR342C	*HXT7*	Glucose, fructose and mannose transporter activity	+2.06
YDR040C	*ENA1*	P-type ATPase sodium pump for sodium ion transport	−2.00
YMR011W	*HXT2*	Glucose, fructose and mannose transporter activity	−2.63
YJR049C	*UTR1*	Protein involved in iron homeostasis	−2.70
YHR094C	*HXT1*	Glucose, fructose and mannose transporter activity	−2.94
Stress response			
YHR106W	*TRR2*	Mitochondrial thioredoxin reductase, response to oxidative stress	−2.17
YDR258C	*HSP78*	Protein involved in folding of some mitochondrial proteins	−2.17
YBR203W	*COS111*	Protein required for resistance to the antifungal ciclopiroxolamine	−2.22
YKR013W	*PRY2*	Protein similar to pathogen related proteins	−2.44
YPL152W	*RRD2*	Protein responding to osmotic stress	−2.63

(*continued on next page*)

Table 1 (*continued*)

ORF	Gene	Description	Fold change
Transcription factor			
YMR270C	*RRN9*	RNA polymerase I upstream activator subunit	+2.15
YGL209W	*MIG2*	Involved in repression of invertase expression by high glucose	−2.13
YFL021W	*GAT1*	Transcriptional activator involved in nitrogen catabolite repression	−2.33
YGL035C	*MIG1*	Transcription factor involved in glucose repression	−2.38
YML099C	*ARG81*	Regulator of arginine-responsive genes	−2.44
Unknown			
YPR002C-A		Protein of unknown function	+2.80
YDR525W-A	*SNA2*	Protein of unknown function	+2.65
YDR396W		Protein required for cell viability	+2.63
YKL102C		Protein of unknown function	+2.39
YMR046W-A		Protein of unknown function	+2.32
YGL029W	*CGR1*	May contribute to compartmentalization of nucleolar constituents	+2.25
YNL156C	*NSG2*	Potential homolog of mammalian Insig 1	+2.16
YDR034C-A		Protein of unknown function	+2.11
YMR118C		Protein of unknown function	+2.10
YBL043W	*ECM13*	Protein of unknown function	+2.09
YGR122C-A		Protein of unknown function	+2.07
YDR051C		Protein of unknown function	+2.06
YBR056W		Protein of unknown function	+2.04
YPL282C		Protein of unknown function	+2.03
YDR033W	*MRH1*	Membrane protein related to Hsp30p	+2.00
YPR091C		Protein of unknown function	−2.00
YNL134C		Protein with alcohol dehydrogenase ($NADP^+$) activity	−2.00
YIL127C		Protein of unknown function	−2.04
YLR108C		Protein of unknown function	−2.04
YKR075C		Protein of unknown function; expression regulated by Rgt1	−2.08
YDR070C	*FMP16*	Found in mitochondrial proteome	−2.17
YFL062W	*COS4*	Subtelomerically encoded protein of unknown function	−2.22
YBR285W		Protein of unknown function	−2.27
YER085C		Protein of unknown function	−2.33
YNR034W-A		Protein of unknown function	−2.38

After a 1-h induction of Bax protein expression, RNA was extracted and used for a comprehensive analysis of the transcript steady-state levels. As a control, the same culture conditions were used for yeast harboring the empty vector. Following RNA extraction, the expression level changes due to Bax expression were determined. Genes for which the expression level changed at least 2-fold due to Bax expression were considered Bax-responsive. Data are represented by the mean of two independent experiments.

Table 2
Validation of the transcript level changes for genes thought to have a Bax-specific transcriptional response

Microarray profiling		Microarray results: Fold change		RT Q-PCR results: Fold change	
ORF	Gene	Bax/Ctrl	H_2O_2/Ctrl	Bax/Ctrl	H_2O_2/Ctrl
YMR297W	*PRC1*	+2.29	−1.33	+1.81	−1.78
YNL117W	***MLS1***	+2.09	−1.22	**+5.75**	+1.18
YDR342C	*HXT7*	+2.06	−1.47	+1.02	−2.70
YIL127C		−2.04	+1.20	+1.89	+3.39
YGR008C	*STF2*	−2.04	+1.09	−1.14	−4.55
YEL039C	*CYC7*	−2.08	+1.15	−1.27	+1.25
YPL250C	***ICY2***	−2.17	−1.06	**−2.38**	+2.02
YJL189W	*RPL39*	−2.22	+2.31	+1.75	+2.48
YPL171C	***OYE3***	−2.22	+1.85	**−2.70**	+1.50
YGR142W	***BTN2***	−2.50	+1.22	**−2.04**	+1.45

A gene was considered transcriptionally responsive to Bax when the transcriptional change elicited by Bax protein expression was at least 2-fold more manifest or was in the opposite sense compared to the oxidative stress trigger. The results obtained for the microarray profiling were compared with the independent RT Q-PCR data. Out of the 10 candidates, four were finally identified to have a Bax-specific transcriptional response. Bold: Genes for which the Bax-specific transcriptional change was verified. Data are represented by the mean of at least two independent experiments.

In addition, the rate of Bax-induced cell death was decreased in Δ*OYE3* (Fig. 2(a)), with a maximal cell death diminution of 18.0% (standard deviation: 4.5%). Bax protein expression in both BY4742 and Δ*OYE3* was verified by Western blot and Bax protein levels were found to be identical for both strains (Fig. 3).

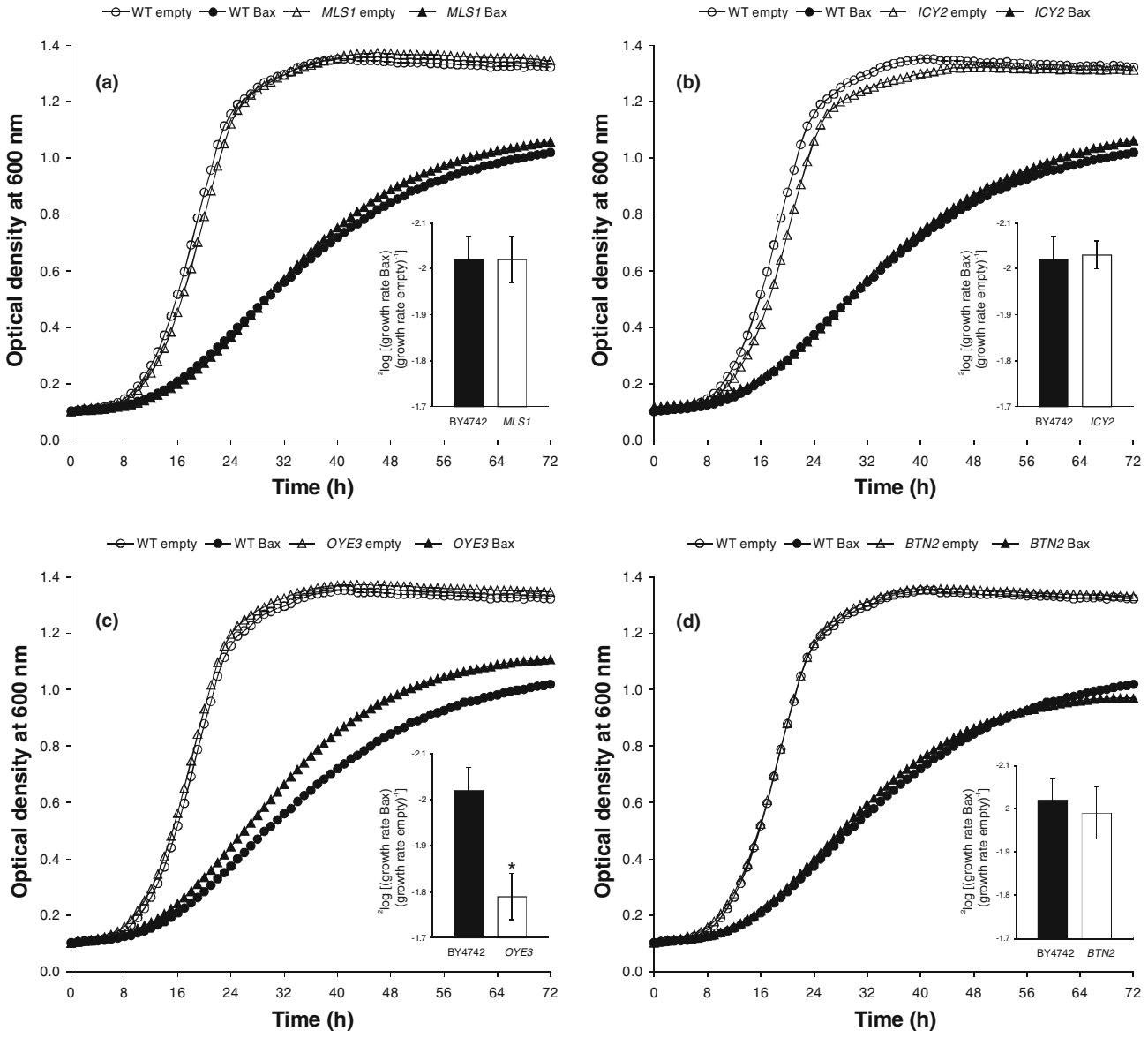

Fig. 1. The rate of Bax-induced growth inhibition is decreased upon deletion of OYE3. Yeast cells were transformed with the plasmids p415GALLmBAX (Bax) or p415GALL (empty), grown in glucose-based medium, and diluted to equal cell densities in galactose-based medium to induce protein expression from the GALL promoter. Growth was monitored automatically over 72 h. Inset: The ratio of the growth rate following Bax protein induction to the control growth rate. Growth in the presence (Bax) and absence (empty) of Bax for (a) Δ*MLS1*, (b) Δ*ICY2*, (c) Δ*OYE3* and (d) Δ*BTN2*, each compared to wild type. Error bars represent the standard deviation of the mean of three independent experiments. (*) Statistically significant difference based on the Wilcoxon Rank Sum test ($\alpha = 0.05$).

3.4. Correlation of Bax- and H_2O_2-induced cell death with a drop in NADPH level

OYE3 encodes an NADPH dehydrogenase for which the physiological substrate is unknown to date. Therefore, the correlation between cell death rate and NADPH levels was investigated. BY4742 and Δ*OYE3* had the same initial NADPH levels (Fig. 4(a)). During Bax-induced cell death NADPH levels decreased, though this NADPH drop was less pronounced in Δ*OYE3* than in BY4742 (Fig. 4(a)). Further, the level of NADPH also decreased during H_2O_2-induced cell death (Fig. 4(c)). In these experiments, the use of 0.5 mM H_2O_2 gave the most reproducible and discriminatory results, although the same trend was observed with lower concentrations. However, contrary to the effects observed during Bax-induced cell death, the absence of *OYE3* resulted in a more prominent drop in the level of NADPH (Fig. 4(c)). As shown in Fig. 2(b), deletion of *OYE3* decreased the survival upon administration of 0.5 mM H_2O_2.

Thus, NADPH levels correlated with the rate of cell death for both cell death triggers.

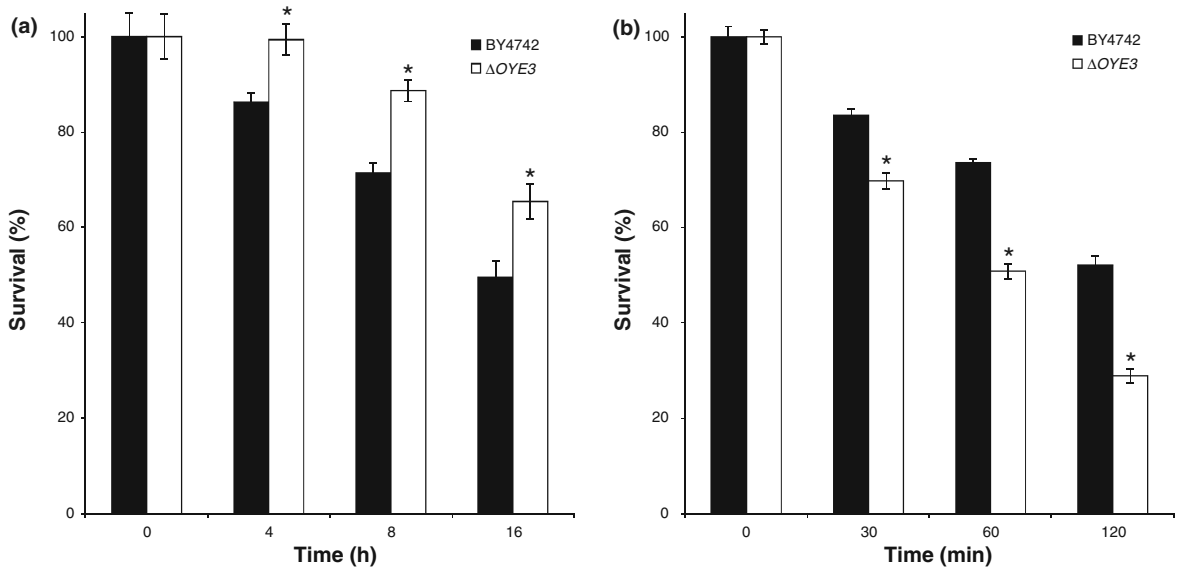

Fig. 2. The absence of *OYE3* has different effects on Bax-induced and H_2O_2-induced cell death. (a) Wild-type BY4742 and Δ*OYE3* cells were transformed with the plasmids p415GALLmBAX (Bax) or p415GALL (empty), grown in glucose-based medium, and recultured in galactose-based medium to induce protein expression from the *GALL* promoter. Clonogenic survival was determined by recovering cells at various times from the galactose-based medium and plating 1000 cells on glucose-based semisolid medium. For each timepoint, the percentage of survival was calculated by dividing the number of colonies surviving the Bax protein induction by the number of colonies appearing after mock-treatment (empty). The absence of *OYE3* attenuates Bax-induced cell death. (b) Wild-type BY4742 and Δ*OYE3* cells were grown in glucose-based medium. Following the addition of H_2O_2 to a final concentration of 0.5 mM, cells were recovered at various times and their clonogenic survival determined. The absence of *OYE3* leads to a sensitivity towards H_2O_2. Error bars represent the standard deviation of the mean of three independent experiments. (*) Statistically significant difference based on the Wilcoxon Rank Sum test ($\alpha = 0.05$).

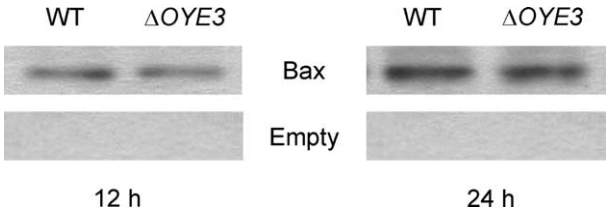

Fig. 3. Bax protein expression is unaffected in yeast cells knocked out for *OYE3*. Wild-type and Δ*OYE3* cells were transformed with the plasmids p415GALLmBAX (Bax) or p415GALL (empty), grown in glucose-based medium, transferred to galactose-based medium and cultured for an additional period of 12 or 24 h. Total protein extracts (30 μg) were subjected to SDS–PAGE and immunoblot analysis, using a monoclonal antibody against murine Bax.

3.5. Lipid peroxidation during Bax-induced cell death is not present in the OYE3 knockout

Based on the observation that the rate of Bax-induced cell death decreases and the rate of H_2O_2-triggered cell death increases upon deletion of *OYE3*, it could be argued that the absence of Oye3 somehow hampered the generation of oxygen stress, providing a protective mechanism against Bax-induced cell death. Therefore, we determined the extent of oxidative damage during Bax-induced cell death in Δ*OYE3* compared to BY4742.

Neither oxidative protein damage nor oxidative mitochondrial DNA damage could be detected following Bax induction (data not shown), indicating that either these molecules are not damaged by the oxidative insult accompanying Bax expression, or else that they are rapidly and efficiently repaired or removed.

Lipid peroxidation, however, was found to be associated with Bax-induced cell death in BY4742 (Fig. 4(b)), but we could not observe any lipid peroxidation in Δ*OYE3* during Bax-induced cell death. These findings implicate Old yellow enzyme Oye3, directly or indirectly, in Bax-induced lipid peroxidation.

Lipid peroxidation was also observed during H_2O_2-induced cell death (Fig. 4(d)), the extent of which was related to the H_2O_2 dose. However, oxidative lipid damage following H_2O_2 treatment was higher in Δ*OYE3* (Fig. 4(d)), in line with the increased H_2O_2 sensitivity (Fig. 2(b)) and the more severe NADPH drop (Fig. 4(c)) upon H_2O_2 addition compared to the wild type.

Overall, these results show that the deletion of *OYE3* clearly interferes with Bax-induced cell death as demonstrated by Bax tolerance, and augments H_2O_2-induced cell death. The different phenotypes induced by the two cell death triggers were reflected in the extent of NADPH decrease and lipid peroxidation in Δ*OYE3*.

4. Discussion

In the present study, we wanted to determine whether the Bax-induced transcriptional responses

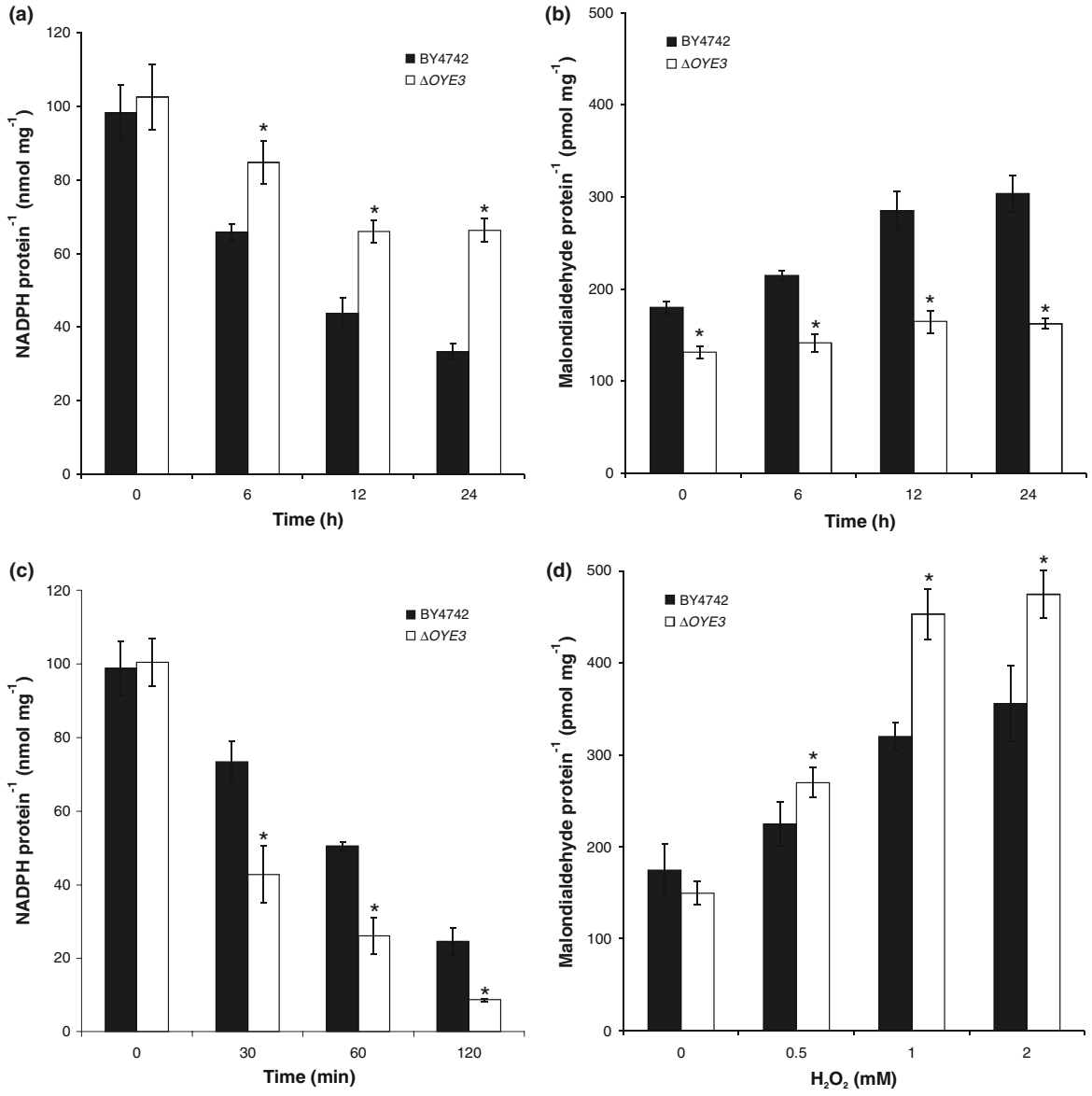

Fig. 4. The absence of *OYE3* has different effects on Bax- and H_2O_2-induced NADPH decrease and increase of thiobarbituric acid-reactive species. Wild-type BY4742 and Δ*OYE3* cells were transformed with the plasmid p415GALLmBAX (Bax), grown in glucose-based medium, and recultured in galactose-based medium. Cells were recovered at various times and assayed (a) for their NADPH levels and (b) for lipid peroxidation breakdown products. The decrease in NADPH level, seen during Bax-induced cell death in wild type, is clearly less pronounced in the strain knocked-out for *OYE3*. The increase in thiobarbituric acid-reactive species, seen during Bax-induced cell death in wild type, is completely absent from the strain knocked out for *OYE3*. (c) Wild-type BY4742 and Δ*OYE3* cells were grown in glucose-based medium. Following the addition of H_2O_2 to a final concentration of 0.5 mM, cells were recovered at various times and assayed for their NADPH levels. The decrease in NADPH level, seen during H_2O_2-induced cell death in wild type, is clearly more pronounced in the strain knocked-out for *OYE3*. (d) Wild-type BY4742 and Δ*OYE3* cells were grown in glucose-based medium. Cells were recovered 1 h following the addition of various concentrations of H_2O_2, and assayed for lipid peroxidation. The increase in thiobarbiturc acid-reactive species, seen during H_2O_2-induced cell death in wild type, is more pronounced in the strain knocked-out for *OYE3*. Error bars represent the standard deviation of the mean of three independent experiments. (*) Statistically significant difference based on the Wilcoxon Rank Sum test ($\alpha = 0.05$).

were different from those elicited by oxidative stress, and if so, whether these differences could explain how Bax expression actually generates a cellular oxidative stress.

First, we performed microarray expression profiling of the early transcriptional responses to Bax expression, and identified 75 Bax-responsive genes. Second, we optimized a H_2O_2 challenge to be as lethal as Bax expression. Due to the high membrane permeability of H_2O_2, its addition to the culture medium was thought to mimic the intracellular ROS generated as a consequence of expressed Bax protein. The tran-

script profiling of the H_2O_2 responses was in agreement with data published by others [84,85]. Third, the transcriptional responses of the Bax-responsive genes to Bax induction and H_2O_2 treatment were compared. Ten of the 75 Bax-responsive genes showed a Bax-associated change that was at least 2-fold more manifest or was in the opposite sense compared to the corresponding H_2O_2-induced change. Fourth, we independently validated the transcriptional responses of these 10 genes by quantitative real-time PCR, and four genes (*MLS1*, *OYE3*, *ICY2* and *BTN2*) were confirmed to have a Bax-specific transcriptional response. Homologues to these genes (*MLS2*, *OYE2* and *ICY1*) did not demonstrate Bax-associated transcriptional changes in this study. Thus, the genes *MLS1*, *OYE3*, *ICY2* and *BTN2* could constitute part of the mechanism either by which Bax elicits oxygen stress or by which cells protect themselves against Bax toxicity. Particularly, the selection of four genes with a Bax-specific response reinforced our view about the difference between Bax expression and H_2O_2 treatment in eliciting yeast cell death.

We analyzed the phenotypic outcome of Bax expression in yeast strains individually knocked out for each of these four genes. We observed that only the absence of *OYE3* (Old yellow enzyme) increased the tolerance to Bax-induced cell death. However, the Δ*OYE3* strain clearly demonstrated H_2O_2 sensitivity. The difference in phenotypes of Δ*OYE3* upon oxidative stress and Bax expression was interpreted as a reflection of the differential transcriptional response of this gene towards the two stresses (Bax: 2.70-fold down, H_2O_2: 1.50-fold up). Moreover, deletion of *OYE3* per se did not increase generation time.

Old yellow enzyme (OYE; EC 1.6.99.1) was originally isolated from brewer's bottom yeast as "das gelbe Ferment" [86], and was shown to be composed of a colorless apoprotein and a redox-active flavin mononucleotide prosthetic group that gives it its distinctive yellow color [87]. Old yellow enzyme reduces the olefinic bond of α, β-unsaturated aldehydes and ketones [88–90], with NADPH as the physiological reductant for the enzyme-bound flavin [91,92].

As Oye3 is an NADPH dehydrogenase, we wondered whether an increased intrinsic NADPH level, due to the absence of an NADPH-converting enzyme in Δ*OYE3*, could be responsible for the increased Bax tolerance. However, the decreased rate of Bax-induced cell death in the absence of *OYE3* could not be attributed to an intrinsically augmented redox buffering capacity. Further, Bax-induced and H_2O_2-induced cell death were accompanied by a drop in NADPH level proportionate to the cell death rate (Fig. 5). Our observation of NADPH consumption during Bax-induced cell death further strengthens the position of oxygen stress as a general regulator of cell death [14], and extends the observation that intracellular glutathione is shifted to the more oxidized state during Bax expression [64].

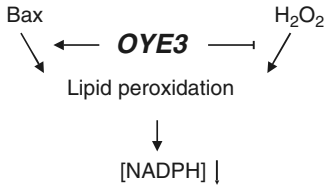

Fig. 5. Old yellow enzyme Oye3 interferes with Bax toxicity. Both Bax expression and H_2O_2 treatment induce lipid peroxidation and NADPH consumption, and these changes are part of both cell death processes. A drop in cellular NADPH will lead to redox buffer shortage and oxidative burden. Peroxidized lipids could affect membrane physiology or may interfere with an unknown signaling mechanism. The absence of Oye3 sensitizes yeast cells to H_2O_2-induced cell death but attenuates Bax-induced cell death. The absence of Oye3 is thought to increase H_2O_2 sensitivity due to the loss of its NADPH dehydrogenase activity, the substrates of which are likely to be lipid peroxidation breakdown products. Oye3 may be involved in the mechanism by which Bax expression leads to oxidative stress. For details, please refer to text.

The decreased rate of Bax-induced cell death and the increased rate of H_2O_2-triggered cell death upon deletion of *OYE3* led us to investigate the extent of oxidative damage during Bax-induced cell death in Δ*OYE3* compared to the wild type. Surprisingly, neither protein nor mitochondrial DNA oxidative damage was observed following Bax expression in wild type.

It has been proposed that the prime physiological substrates for Old yellow enzyme may be lipid peroxidation breakdown products [92], which can be toxic to cells [93], and are known to be potent apoptotic triggers [94–97]. Lipid peroxidation was found to be associated with Bax-induced cell death, in agreement with another study [44], and with H_2O_2-induced cell death (Fig. 5). Due to the absence of *OYE3*, no lipid peroxidation was observed during Bax-induced cell death, but lipid peroxidation breakdown products accumulated to a higher level following H_2O_2 treatment. The latter result is in agreement with the observation that *OYE3* is part of the Yap1 regulon [85,98–100].

In conclusion, our results clearly indicate that Oye3 is involved, directly or indirectly, in Bax-induced lipid peroxidation and the drop in NADPH level. This suggests Oye3 may interfere with the mechanism by which Bax expression leads to oxidative stress, being a part of the yeast-specific response to Bax-induced cell death.

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