***N*-Myristoylation of Arf proteins in *Candida albicans*: an *in vivo* assay for evaluating antifungal inhibitors of myristoyl-CoA:protein *N*-myristoyltransferase**

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Myristoyl-CoA:protein *N*-myristoyltransferase (Nmt) catalyses the covalent attachment of myristate to the N-terminal glycine of a small subset of cellular proteins produced during vegetative growth of *Candida albicans*. *nmt447D* is a mutant *NMT* allele encoding an enzyme with a Gly⁴⁴⁷ → Asp substitution and reduced affinity for myristoyl-CoA. Among isogenic *NMT/NMT*, *NMT/Δnmt* and *nmtΔ/nmt447D* strains, only *nmtΔ/nmt447D* cells require myristate for growth on yeast/peptone/dextrose media (YPD) at 24 or 37 °C. When switched from YPD/myristate to YPD alone, 60% of the organisms die within 4 h. Antibodies raised against the C-terminal eight residues of *Saccharomyces cerevisiae* Arf1p were used to probe Western blots of total cellular proteins prepared from these isogenic *Candida* strains. *N*-Myristoylation of *C. albicans* ADP-ribosylation factor (Arf) produced a change in its electrophoretic mobility during SDS-PAGE: the myristoylated species migrated more rapidly than the nonmyristoylated species. In an *NMT/nmtΔ* strain, 100% of the Arf is *N*-myristoylated based on this mobility shift assay. When exponentially growing *nmtΔ/nmt447D* cells were incubated at 24 °C in YPD/myristate, < 25% cellular Arf was nonmyristoylated. In contrast, 2 or 4 h after withdrawal of myristate, ≥ 50% of total cellular Arf was nonmyristoylated. This finding suggests that ≥ 50% reduction in Arf *N*-myristoylation is a biochemical marker of a growth-arrested cell. A similar conclusion was made after assaying isogenic *S. cerevisiae* strains containing various combinations of *NMT1*, *nmt1-451D*, *ARF1*, *arf1Δ*, *ARF2* and *arf2Δ* alleles and grown at 24–37 °C on YPD or YPD/myristate. Peptidomimetic inhibitors of *C. albicans* Nmt were synthesized based on the N-terminal sequence of an *S. cerevisiae* Arf. SC-59383 has an IC₅₀ of 1.45 ± 0.08 μM for purified *C. albicans* Nmt and is 560-fold selective for the fungal compared to human *N*-myristoyltransferase. It had an EC₅₀ of 51 ± 17 and 67 ± 6 μM, 24 and 48 h after a single administration of the drug to cultures of *C. albicans*. The Arf gel mobility shift assay indicated that a single dose of 200 μM produced a < 50% reduction in Arf *N*-myristoylation after 4 h, which is consistent with the fungistatic, but not fungicidal, activity. The effect on Nmt was specific: an enantiomer, SC-59840, had no inhibitory effect on purified *C. albicans* Nmt (IC₅₀ > 1000 μM), and 200 μM of the compound produced no detectable reduction in Arf *N*-myristoylation *in vivo*. SC-58272, which is related to SC-59383, was a more potent inhibitor *in vitro* (IC₅₀ 0.056 ± 0.01 μM), but had no growth inhibitory activity and did not produce any detectable reduction in Arf

N-myristoylation. These findings highlight the utility of the Arf protein gel mobility shift assay for demonstrating the mechanism-based antifungal activity of SC-59383, a selective inhibitor of *C. albicans* Nmt.

Keywords: protein N-myristoylation, ADP-ribosylation factors, *Candida albicans*, *Saccharomyces cerevisiae*, antifungal agents

INTRODUCTION

Protein N-myristoylation refers to the co-translational covalent linkage of myristate, a 14-carbon saturated fatty acid (C14:0), to the N-terminal glycine of eukaryotic proteins. This protein modification appears to be irreversible and is catalysed by myristoyl-CoA:protein N-myristoyltransferase (Nmt). Nmt substrates have a variety of biological functions and include proteins involved in signal transduction pathways (kinases and their substrates, phosphatases, α -subunits of heterotrimeric G proteins, NO synthase), vesicular trafficking within the Golgi apparatus (ADP-ribosylation factors, Arfs), as well as a number of structural and nonstructural proteins encoded by enveloped and nonenveloped viruses (reviewed by Johnson *et al.*, 1994a; Chow & Moscufo, 1995).

Several observations indicate that Nmt may be an attractive target for development of a new class of fungicidal agents for treating immunocompromised patients, including those with AIDS. Nmt is encoded by a single copy gene in *Candida albicans* and *Cryptococcus neoformans* (Wiegand *et al.*, 1992; Lodge *et al.*, 1994a). Conditional lethal *nmt* alleles have been introduced into *C. albicans* and *C. neoformans* genomes by homologous recombination (Weinberg *et al.*, 1995; Lodge *et al.*, 1994b). These alleles specify Nmts with reduced affinity for myristoyl-CoA owing to substitution of a conserved Gly, located five amino acids from their C-terminus, with an Asp. The mutant *nmt* alleles produce temperature-sensitive growth arrest and myristic acid auxotrophy. Withdrawal of myristate from the medium results in cell death, thereby establishing that Nmt is essential in these two organisms. In addition, an immunosuppressed rabbit model of human *C. neoformans* meningitis and an immunosuppressed mouse model of systemic *C. albicans* infection were used, together with isogenic strains of fungi with either wild-type or the conditional lethal *nmt* allele, to demonstrate that (genetic) attenuation of Nmt activity allows the immunocompromised host to cure itself of the fungal infection (Lodge *et al.*, 1994b; Weinberg *et al.*, 1995).

In addition to genetic proof for the concept that Nmt is a target for development of fungicidal drugs, selective species-specific Nmt inhibitors have been produced (Devadas *et al.*, 1995, 1996). The enzyme has an ordered bi bi reaction mechanism (Rudnick *et al.*, 1991; Rocque *et al.*, 1993; Bhatnagar *et al.*, 1994). It binds myristoyl-CoA forming a binary complex. Conformational changes associated with ligation result in generation of a functional peptide-binding site and formation of a ternary myristoyl-CoA:Nmt:peptide

complex. Catalysis and product release then occur. The myristoyl-CoA-binding sites of purified human Nmt and the fungal Nmts are highly conserved, but their peptide-binding specificities are quite divergent (Lodge *et al.*, 1994b; Devadas *et al.*, 1995). Starting with a high-affinity octapeptide substrate of *C. albicans* Nmt, dipeptide inhibitors of the fungal enzyme have been synthesized that are competitive for binding of peptide substrates but not myristoyl-CoA. Several potent inhibitors (K_i 30–60 nM) are selective (up to 400-fold) for the *Candida* enzyme compared to the human enzyme *in vitro* (Devadas *et al.*, 1995, 1996).

The effects of members of this class of inhibitors on fungal growth and viability have not been reported. In this work, we describe development of an *in vivo* assay for protein N-myristoylation in *C. albicans* and its use in assessing the efficacy of Nmt inhibitors.

METHODS

Strains and media. *Saccharomyces cerevisiae* strains YB332 (NMT1) and YB336 (*nmt1-451D*) are isogenic (Johnson *et al.*, 1994b). *S. cerevisiae* strains TT104 (NMT1 *arf1Δ::HIS3* ARF2; Kahn *et al.*, 1995) and TT139 (NMT1 *arf1Δ::LEU2*) are also isogenic and were obtained from Richard Kahn (Emory University, Atlanta, GA, USA). The methods used to construct the isogenic *C. albicans* strains MONCA105 (NMT/*nmtΔ*) and MONCA106 (*nmt447D/nmtΔ*) are detailed in Weinberg *et al.* (1995). *C. albicans* strain B311 (NMT/NMT) is a previously characterized clinical isolate, obtained from George Kobayashi (Washington University, USA), which was used to isolate the fungus' NMT as well as one of its ARF genes (Wiegand *et al.*, 1992; Langner *et al.*, 1992).

YPD medium contained 1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco) and 2% (w/v) dextrose. YPD/myristate contained 500 μ M myristate (Nuchek Prep) and 1% Brij-58 (Sigma). Yeast Nitrogen Base broth (YNB) was obtained from Difco.

Construction of *S. cerevisiae* *arf1Δ* or *arf2Δ* strains isogenic to YB332 and YB336. DNA fragments containing *arf1Δ::HIS3* or *arf2Δ::LEU2* were obtained by PCR using genomic DNA from *S. cerevisiae* strain TT104 (*arf1Δ::HIS3*) or TT139 (*arf2Δ::LEU2*) as templates, 5'-CGAAAAGATAAGTATCTCGCTCAACC and 5'-CAAATGAAAATGCGTTGTATAATATCC (*arf1Δ::HIS3*) or 5'-GATTATCTAATAGGGCCTAGTTATCC and 5'-GCTAGTAAGTTTATTTTCAGTGCACC (*arf2Δ::LEU2*) as primers, the *Taq* polymerase KTLA and its recommended buffer (Barnes, 1994) plus the following cycling conditions: 94 °C for 15 s, 55 °C for 30 s, 72 °C for 5 min, repeated 25 times. PCR fragments were purified by agarose gel electrophoresis and transformed (Gietz *et al.*, 1995) into YB332 and YB336. The resulting strains, YB535 (NMT1 *arf1Δ::HIS3* ARF2), YB539 (*nmt1-451D* *arf1Δ::HIS3* ARF2), YB537 (NMT1 ARF1 *arf2Δ::LEU2*) and YB541

(*nmt1-451D ARF1 arf2Δ::LEU2*), were prototrophic for either histidine or leucine. Replacement of their wild-type *ARF1* or *ARF2* genes with the corresponding *arf* null alleles was confirmed by PCR using the primers listed above.

Generation of *N*-myristoylated and nonmyristoylated Arf standards. A dual plasmid expression system was used to produce *N*-myristoylated *S. cerevisiae* Arf1p or Arf2p in *Escherichia coli* strain JM101 (Lodge *et al.*, 1994a). This system consists of two recombinant plasmids with *NMT1* or *ARF* inserts under the control of different inducible promoters. Nmt1p expression is initiated in exponentially growing bacteria followed 30 min later by induction of an Arf substrate. The corresponding nonmyristoylated Arfs were obtained by simply omitting the expression vector encoding Nmt1p (note that *E. coli* has no endogenous Nmt activity). Bacterial lysates were prepared (Lodge *et al.*, 1994a) and used as standards for a protein mobility gel shift assay described below. A similar strategy was used to produce *N*-myristoylated and nonmyristoylated forms of a *C. albicans* Arf originally cloned from strain B311 (Langner *et al.*, 1992).

A protein mobility assay for examining cellular levels of *N*-myristoylated and nonmyristoylated Arf proteins. Cultures (50 ml) of *S. cerevisiae* strains YB332 (*NMT1 ARF1 ARF2*), YB336 (*nmt1-451D ARF1 ARF2*), YB535 (*NMT1 arf1Δ ARF2*), YB537 (*NMT1 ARF1 arf2Δ*), YB539 (*nmt1-451D arf1Δ ARF2*) and YB541 (*nmt1-451D ARF1 arf2Δ*), or *C. albicans* strains MONCA105 (*NMT/nmtΔ*) and MONCA106 (*nmt447D/nmtΔ*), were grown overnight at a permissive temperature in YPD/myristate to an $OD_{600} \approx 2.0\text{--}3.0$ (measured with an KB Ultrospec II spectrophotometer). Cells were collected by centrifugation at room temperature. Each cell pellet was washed three times with 25 ml PBS (0.145 M NaCl; 0.15 M sodium phosphate, pH 7.4), and resuspended in 4–6 ml YPD. A 1 ml aliquot was added to 10 ml YPD or YPD/myristate and incubated at 24, 30 or 37 °C for 2 or 4 h. Cells were then pelleted as above, washed once in 5 ml PBS, and resuspended in 0.5 ml lysis buffer (4% w/v, SDS; 0.125 M Tris/HCl, pH 6.8). An equal volume of glass beads (425–600 µm diameter; Sigma) was added, and the suspension was vortexed for 1 min alternating with ice for 1 min for a total of three cycles. The lysates were then boiled for 5 min and cellular debris was removed by centrifugation at 10000 *g* for 10 min. The protein concentrations of the resulting supernatants were determined with the BCA kit (Pierce). Following addition of glycerol, β -mercaptoethanol and bromophenol blue to final concentrations of 5% (v/v), 2% (v/v) and 0.002% (w/v), respectively, samples of cellular proteins (10 µg in the case of *S. cerevisiae* lysates; 100 µg with *C. albicans* lysates) were fractionated by electrophoresis through 12% acrylamide gels (16 cm long and 0.75 mm thick for *S. cerevisiae* lysates or 1.5 mm thick for *C. albicans* lysates) containing 0.1% SDS (Laemmli, 1970). The separated proteins were then transferred to Hybond-PVDF membranes (Amersham) by electroblotting (16 h at 50 mA; transfer buffer was 1.6 mM Tris, 19 mM glycine, 2% v/v, methanol).

Arfs were identified using the protocol described in the Western-Light kit (Tropix) with one modification: the blocking buffer consisted of PBS supplemented with 1% (w/v) gelatin (Sigma), 0.2% (v/v) Tween 20 (Sigma) and 0.1% (w/v) sodium azide (Sigma). Arf1p was detected in *S. cerevisiae* lysates using rabbit antiserum R23, which was raised against a peptide encompassing the C-terminal eight residues of the protein (SNSLKNST¹⁸¹). Arf2p was identified with rabbit antiserum R29 generated using an *S. cerevisiae* Arf2p C-terminal peptide (SNNLNQS¹⁸¹). Both antisera were supplied by R. Kahn and were employed at a final

dilution of 1:20000 (for *S. cerevisiae* lysates) or 1:4000 (for *C. albicans* lysates) in blocking buffer. All Arf mobility assays were repeated at least three times using independently derived cell lysates.

Characterization of dipeptide inhibitors of *C. albicans* Nmt

Determination of IC_{50} for *C. albicans* and human Nmts *in vitro*. Synthesis of the dipeptide Nmt inhibitor SC-58272 is described in an earlier report (compound 17 in Devadas *et al.*, 1995). Details concerning syntheses of SC-59383 and its enantiomer SC-59840 will be published elsewhere. The purified compounds exhibited spectral and analytical data consistent with the structures shown.

IC_{50} values were calculated using (i) *C. albicans* B311 and human Nmts that had been produced in *E. coli* and purified to apparent homogeneity according to previously described protocols (Wiegand *et al.*, 1992; Rudnick *et al.*, 1992), and (ii) a radiochemical HPLC end-point assay (Rudnick *et al.*, 1992; Devadas *et al.*, 1995). The Nmt assay mixture (final volume 60 µl) contained variable amounts of the peptidomimetic inhibitor, 0.11 nmol [³H]myristoyl-CoA [1 µCi (37 kBq); 9 Ci mmol⁻¹ (333 GBq mmol⁻¹)], a substrate peptide (GNAASARR-NH₂; 2.2 nmol when using *C. albicans* Nmt, 0.22 nmol with the orthologous human *N*-myristoyl-transferase), Nmt (10 and 75 ng of the purified *E. coli*-derived fungal and human enzymes, respectively), 0.2 M HEPES (pH 7.4), 2 mM DTT and 0.2 mM EDTA. Note that inhibitors were prepared in DMSO and the final concentration of DMSO in each assay was 0.5% (v/v). All assays were performed on at least three occasions, each time in duplicate. Results are presented as mean values $\pm$ 1 SD.

Determination of EC_{50} values of Nmt inhibitors. Peptidomimetic inhibitors were prepared as 20 mM stock solutions in DMSO and diluted into sterile deionized water (DMSO concentration = 1%, v/v) to yield 2 $\times$ the final desired concentration for the culture-based assay. Portions (100 µl) of the 2 $\times$ stocks were dispensed into triplicate wells of a 96-well microtitre plate (Costar). *C. albicans* strain B311 (a 100 µl portion containing 10⁵ cells in 2 $\times$ YNB) was distributed into each of the wells. Controls included DMSO alone (final concentration = 0.5%; negative control) and amphotericin B (final concentration = 0.05, 1 and 5 µg ml⁻¹; positive control). Plates were incubated at 30 °C and growth was scored by monitoring the OD_{490} using an ELISA plate reader (Dynatech). EC_{50} , the concentration of compound inhibiting growth by 50% compared to untreated controls, was calculated at 24 and 48 h. All experiments were done in triplicate on at least three occasions. Within a given experiment, the triplicate determinations varied by < 10% per sample per time-point.

RESULTS

Defining the levels of *N*-myristoylation of cellular Arfs in *S. cerevisiae* strains with wild-type and mutant *NMT1* alleles

Temperature-sensitive mutants of *NMT1* have proven to be valuable tools for studying the regulation of protein *N*-myristoylation in *S. cerevisiae* (Duronio *et al.*, 1991; Johnson *et al.*, 1993, 1994b). As in *C. albicans*, substitution of an Asp for the conserved Gly⁴⁵¹ located five residues from the C-terminus of the enzyme causes a temperature-sensitive reduction in the affinity of nmt451Dp for myristoyl-CoA. This is associated with global defects in cellular protein *N*-myristoylation (Duronio *et al.*, 1991). The *S. cerevisiae* *nmt1-451D*

Fig. 1. Antibodies that specifically react with *S. cerevisiae* Arf1p and Arf2p. Duplicate Western blots were probed with rabbit antibodies raised against peptides representing the C-terminal eight residues of Arf1p or Arf2p. Lanes 1 and 2 (standards) of each blot contain 100 ng cellular lysates prepared from *E. coli* strains that co-express Nmt1p and either *S. cerevisiae* Arf1p (lanes 1) or Arf2p (lanes 2). Lanes 3, 4 and 5 contain 10 μ g lysate proteins prepared from isogenic *S. cerevisiae* strains YB332 (*NMT1 ARF1 ARF2 ARF3*), YB535 (*NMT1 arf1 Δ ARF2 ARF3*) and YB537 (*NMT1 ARF1 arf2 Δ ARF3*), respectively.

strain has no growth defects on rich media at 24 °C. Growth arrest occurs within 1 h after shifting to 30–37 °C. Cells remain viable at 37 °C for 8 h. Death begins within 12 h, and no viable cells are detectable after 24 h (Johnson *et al.*, 1994b). Steady state levels of Nmt1p and nmt451Dp are comparable in isogenic strains 1 and 4 h after shifting to 37 °C (Johnson *et al.*, 1994b).

Isogenic *NMT1* and *nmt1-451D* strains were used to develop an assay for assessing levels of cellular protein *N*-myristoylation. *S. cerevisiae* contains two principal Arf proteins, Arf1p and Arf2p. These proteins are both *N*-myristoylated, require myristate for expression of their essential biological function(s), and are interchangeable (Stearns *et al.*, 1990; Kahn *et al.*, 1995; Randazzo & Kahn, 1995). We had previously noted that *N*-myristoylation of Arf1p or Arf2p changes their electrophoretic mobility during SDS-PAGE: myristoyl-Arfp, produced in *E. coli* containing expression vectors encoding Nmt1p and one of the Arfs, migrates slightly faster during SDS-PAGE than nonmyristoylated Arf1p produced in bacteria containing only the Arfp expression vector (Lodge *et al.*, 1994a). This protein mobility gel shift formed the basis for monitoring protein *N*-myristoylation in *nmt1-451D* cells during incubation at permissive and nonpermissive temperatures.

Although Arf1p and Arf2p are 96% identical, the C-termini are not well conserved. A rabbit antiserum raised against a peptide representing the C-terminus of Arf1p (SNSLKNST) detects Arf1p produced in *E. coli* but not *E. coli*-derived Arf2p (Fig. 1). Similarly, a rabbit antiserum generated against the C-terminal eight residues of Arf2p (SNNLKNQS) recognizes Arf2p but not Arf1p (Fig. 1). Western blots of bacterial lysates demonstrated that each antibody preparation recognizes the *N*-myristoylated and nonmyristoylated forms of the corresponding recombinant Arf protein (Figs 1 and 2).

S. cerevisiae contains a third *ARF* gene encoding an Nmt1p substrate (Lee *et al.*, 1994). The specificity of the Arf1p- and Arf2p-peptide-specific antibodies was further substantiated using a panel of isogenic *S. cerevisiae* *NMT1* strains with wild-type and null alleles of either *ARF1* or *ARF2*. The Arf1p peptide antibodies

do not recognize any proteins in Western blots of lysates prepared from *NMT1 arf1 Δ ARF2 ARF3* cells harvested during exponential growth in YPD (Fig. 1). Likewise, the Arf2p peptide antibodies do not recognize any proteins in lysates prepared from isogenic *NMT1 ARF1 arf2 Δ ARF3* cells incubated under the same conditions (Fig. 1).

The Arf-specific antibodies were subsequently used to compare the state of Arf1p and Arf2p *N*-myristoylation in isogenic strains containing all possible combinations of *NMT1*, *nmt1-451D*, *ARF1*, *arf1 Δ* , *ARF2* and *arf2 Δ* alleles. In *NMT1* strains, only a single Arf1p or Arf2p species was observed during the mid-exponential phase of growth in YPD at 24, 30 or 37 °C (Fig. 2 and data not shown). The immunoreactive bands detected in protein blots of *S. cerevisiae* cell lysates co-migrated with *E. coli*-derived *N*-myristoylated Arf1p or *N*-myristoylated Arf2p standards (Fig. 2 and data not shown). These findings indicate that both Arf1p and Arf2p are fully myristoylated in *NMT1* cells under these growth conditions.

The *nmt1-451D ARF1 ARF2* strain was also grown to mid-exponential phase at the permissive temperature and then incubated for an additional 2 h at 24, 30 or 37 °C in YPD or YPD/myristate. In the presence of YPD/myristate, there was a progressive increase in the amount of nonmyristoylated Arf1p or Arf2p species as the temperature was raised from 24 to 37 °C, although the percentage did not exceed 50% of total cellular Arf1p or total Arf2p (Fig. 2). In the absence of myristate, nonmyristoylated Arf1p and Arf2p reached 50% of the total at the temperature where cells underwent growth arrest (see lanes marked 37 °C in Fig. 2). Note that two criteria were used to identify the nonmyristoylated forms of Arf1p and Arf2p: (i) their reaction with peptide-specific antibodies; and (ii) their co-migration with the recombinant nonmyristoylated Arf1p and Arf2p, which themselves have distinct mobilities.

Similar experiments using isogenic *nmt1-451D* strains with *arf1 Δ* or *arf2 Δ* null alleles and the Arf-peptide-specific antibodies confirmed that there is an equivalent increase in the fractional representation of nonmyristoylated Arf1p and Arf2p as the temperature is increased. Moreover, supplementation of the medium

Fig. 2. Arf protein gel mobility shift assay for defining cellular levels of protein N-myristoylation in *S. cerevisiae* strains with wild-type and mutant *NMT1* alleles. Western blots were probed with the Arf1p-peptide-specific or Arf2p-peptide-specific antibodies. The first panel contains 100 ng cellular proteins from *E. coli* strains producing either nonmyristoylated or N-myristoylated Arf1p (designated Arf1p and myr-Arf1p standards, respectively). Note the increased mobility of N-myristoylated Arf1p compared to its nonmyristoylated form. The next two panels show blots of yeast cellular lysates (10 µg protein per lane) prepared from various isogenic strains after a 2 h incubation at the indicated temperature in YPD with (+) or without (–) 500 µM myristate. These blots were each probed with the Arf1p-specific antibodies. The three panels on the right of the figure represent blots that were probed with Arf2p-specific antibodies. Standards consist of lysates (100 ng protein per lane) prepared from *E. coli* strains that express Arf2p with or without Nmt1p. Arf2p is fully N-myristoylated in *NMT1* *ARF1* *ARF2* and *NMT1* *arf1Δ* *ARF2* cells at 24–37 °C in YPD or YPD/myristate (data not shown). The relative ratios of N-myristoylated and nonmyristoylated Arf2p in *nmt1-451D* *ARF1* *ARF2* and *nmt1-451D* *arf1Δ* *ARF2* cells at 24 and 30 °C (data not shown) are equivalent to the ratios of N-myristoylated and nonmyristoylated Arf1p in *nmt1-451D* *ARF1* *ARF2* cells (shown).

with myristate produces a comparable ‘return’ of the N-myristoylated forms of both Arfs (Fig. 2 plus data not shown).

The results presented in Fig. 2 allowed us to conclude that both Arf1p and Arf2p exhibit a temperature-dependent reduction in N-myristoylation in *nmt1-451D* but not *NMT1* cells. Although this reduction occurs within 2 h (i.e. 6 h prior to the onset of cell death), an increase in the representation of either nonmyristoylated Arf1p or nonmyristoylated Arf2p to $\geq 50\%$ of total cellular Arf1p or Arf2p appears to be a biochemical marker of a jeopardized, growth-arrested cell.

Assessment of cellular protein N-myristoylation in isogenic *NMT* and *nmt447D* strains of *C. albicans* using the Arf protein gel mobility shift assay

Arfs are the most prominently labelled N-myristoylproteins observed when cultures of wild-type *C. albicans* are incubated with [3 H]myristate during exponential growth in rich media (Langner *et al.*, 1992). One *ARF* gene has been recovered from this organism (Langner *et al.*, 1992; Denich *et al.*, 1992). Expression of its protein product, with or without *C. albicans* Nmt, in *E. coli* cells confirmed that this Arf is a substrate for the N-myristoyltransferase and that covalent attachment of myristate produces an increase in its mobility on SDS-PAGE (Lodge *et al.*, 1994a). Southern blots and PCR studies indicate that *C. albicans* contains at least one other conserved *ARF* (Denich *et al.*, 1992; J. Lodge, unpublished observations).

The *S. cerevisiae* Arf1p-peptide-specific antiserum recognizes a ~20 kDa protein produced in the *NMT*/

nmtΔ strain of *C. albicans* (Fig. 3). This protein has the same mobility on SDS-PAGE as (i) the Arf(s) which are metabolically labelled in the fungus by [3 H]myristate (cf. Langner *et al.*, 1992); and (ii) N-myristoylated recombinant *C. albicans* Arf. The Arf2p-peptide-specific antibody does not react with any *C. albicans* cellular proteins (data not shown). The fungal protein recognized by the Arf1p antiserum is not the same protein encoded by the known *C. albicans* *ARF* gene: Western blots established that the Arf1p-peptide-specific antibodies do not react with the *C. albicans* Arf produced in *E. coli*, whether or not it contains an N-terminal myristoyl moiety (data not shown). Moreover, the C-termini of the *S. cerevisiae* Arf1p and the cloned *C. albicans* Arf are quite divergent (Arf1p = SNSLKNST; *C. albicans* Arf = SNQVGK). These findings suggest that Arf1p-peptide-specific antiserum recognizes a protein encoded by another *C. albicans* *ARF* gene.

NMT/*nmtΔ* and *nmt447D*/*nmtΔ* strains of *C. albicans* were grown in YPD/myristate at 24 °C to mid-exponential phase. Cells were then transferred to YPD with or without 500 µM myristate and incubated at 24 or 37 °C for an additional 2 or 4 h. Western blots of cell lysates were probed with the Arf1p-peptide-specific antiserum. A single immunoreactive species was identified in *NMT*/*nmtΔ* cells under all growth conditions and at both time-points (Fig. 3 and data not shown). In contrast, a second, more slowly migrating immunoreactive protein species appeared in lysates prepared from *nmt447D*/*nmtΔ* cells. In the presence of myristate, this second, slowly migrating species was less abundant than the more rapidly migrating protein that co-migrated with the single band present in *NMT*/*nmtΔ*

Fig. 3. Differing levels of Arf *N*-myristoylation in isogenic *C. albicans* strains with wild-type and conditional lethal *NMT* alleles. Exponential-phase *NMT/nmtΔ* and *nmt447D/nmtΔ* cells were incubated for 2 h at the indicated temperatures in YPD or YPD supplemented with 500 μ M myristate. Cell lysates containing 100 μ g protein were fractionated by SDS-PAGE and the blot was probed with antibodies raised against the *S. cerevisiae* Arf1p C-terminal peptide. Similar ratios of *N*-myristoylated and nonmyristoylated Arf were noted in *nmt447D/nmtΔ* cells after a 4 h incubation under various conditions (data not shown).

	IC ₅₀			EC ₅₀	
	CaNmt	HNmt	Selectivity	24 h	48 h
SC-58272	0.056 $\pm$ 0.01 μ M	14.1 $\pm$ 4.25 μ M	251-fold	>100 μ M	>100 μ M
SC-59383	1.45 $\pm$ 0.08 μ M	809.5 $\pm$ 33.2 μ M	558-fold	51 $\pm$ 17 μ M	67 $\pm$ 6 μ M
SC-59840	>1000 μ M	>1000 μ M	—	>100 μ M	>100 μ M

Fig. 4. Structures, and *in vitro* and *in vivo* activities of peptidomimetic compounds. IC₅₀ values were determined using purified *E. coli*-derived human and *C. albicans* (strain B311) Nmts (HNmt and CaNmt, respectively). Selectivity refers to the ratio of the IC₅₀ for the fungal:human enzyme. EC₅₀ values were determined at 24 and 48 h after administration of single doses of the compound to exponential-phase cultures of *C. albicans* strain B311.

cells. The fractional representation of the more slowly migrating species increases with increasing temperature (Fig. 3). In the absence of myristate, the more slowly migrating immunoreactive protein becomes predominant, increasing within 2 h to > 50% at 24 °C and ~ 90% of the total at 37 °C (Fig. 3). Comparable values were noted 4 h after withdrawal of myristate (data not shown). It is important to note that *nmt447D/nmtΔ* cells require supplementation of YPD with myristate for growth at 24 °C and begin to die within 4 h after removal of myristate (Weinberg *et al.*, 1995).

Based on these results, we concluded that a temperature-dependent increase in the more slowly migrating protein

reflected an increase in the fractional representation of nonmyristoylated Arf. Attenuation of Nmt activity to the point where $\geq$ 50% of an Arf species is nonmyristoylated appears to be associated with growth arrest and loss of viability.

Use of the Arf protein mobility shift assay to study the biological effects of peptidomimetic inhibitors of *C. albicans* Nmt

A high-affinity octapeptide substrate for *C. albicans* Nmt (GLYASKLS-NH₂; *K_m* 0.6 μ M), representing the N-terminal sequence of Arf2p, was used as the starting point for synthesis of peptidomimetic inhibitors of the

Fig. 5. Effects of peptidomimetic compounds on Arf N-myristoylation in *C. albicans*. Cells were treated with a single 200 μ M dose of the compound during exponential-phase growth in YPD at 24 °C. Lysates were prepared 2 and 4 h after drug administration. Blots containing 100 μ g cellular protein per lane were probed with the Arf1p-peptide-specific antibodies. The lane marked 'standard' contains 100 μ g proteins prepared from *nmt447D/nmtΔ* cells incubated at 24 °C. The arrowheads point to the nonmyristoylated form of Arf, which only appears in the standard lane and in cells treated with SC-59383.

enzyme. Substitution of Ala for Gly¹ yielded the competitive inhibitor ALYASKLS-NH₂. Amino acids 1–4 were then replaced with a *para*-(2-methylimidazole-*N*-butyl) phenylacetic acid derivative, amino acids 7 and 8 (Leu-Ser-NH₂) were deleted, and the 2-cyclohexylethyl amide derivative of lysine was used as the C-terminal residue. The structure of the resulting dipeptide inhibitor containing L-serine and L-lysine is presented in Fig. 4 as SC-58272 (cf. Devadas *et al.*, 1995). SC-58272 is a potent inhibitor of purified *C. albicans* Nmt with an IC₅₀ of 56 nM. It is competitive for binding of peptide substrates but not myristoyl-CoA (Devadas *et al.*, 1995) and 250-fold selective for the fungal enzyme compared to human enzyme (see Fig. 4). However, SC-58272 has no growth inhibitory effects on *C. albicans* cultured in YNB medium (EC₅₀ > 100 μ M) (Fig. 4). *NMT/nmtΔ* cells were exposed for 2 or 4 h to 200 μ M SC-58272 during exponential-phase growth. The Arf protein gel shift assay revealed no effect on Arf N-myristoylation (Fig. 5). These findings suggest that SC-58272 is either metabolically unstable or does not gain access to Nmt within cells.

Introduction of a carboxyl group geminal to the lysine amide nitrogen in SC-58272 yielded a tripeptide inhibitor containing L-serine, L-lysine and a cyclohexyl-L-alanine at its C-terminus (SC-59383 in Fig. 4). This compound is a less potent inhibitor of *C. albicans* Nmt than SC-58272 (IC₅₀ 1.45 $\pm$ 0.08 μ M) but is more selective for the fungal enzyme compared to human enzyme (560-fold versus 250-fold for SC-58272; see Fig. 4). Despite its more modest inhibitory activity *in vitro*, a single dose of 100 μ M SC-59383 produced marked suppression of growth for 24 h (Fig. 6a) (EC₅₀ 51 $\pm$ 17 μ M; Fig. 4). The effect was fungistatic rather than fungicidal. The effectiveness of a single dose of the compound diminishes with time (Fig. 6a). Addition of 100 μ M doses of the compound at 12 h intervals produced a sustained

suppression of growth but not cell death (Fig. 6b). The Arf protein gel shift assay indicated that a single dose of 200 μ M of the compound produces a modest reduction in Arf N-myristoylation within 2–4 h (Fig. 5).

Further evidence that the fungistatic effect of SC-59383 is due to its Nmt inhibitory activity comes from an analysis of its enantiomer, SC-59840. Unlike SC-59383, SC-59840 contains D-serine, D-lysine and a C-terminal cyclohexyl-D-alanine. Substitution of these D- for L-amino acids abolishes Nmt inhibitory activity *in vitro* (IC₅₀ > 1000 μ M), providing the first demonstration of the preference of *C. albicans* Nmt for L-amino acids at these positions. SC-59840 has no effect on cell growth (EC₅₀ > 100 μ M at 24 and 48 h; Figs 4 and 6b), even at concentrations up to 1000 μ M (Fig. 6c). Moreover, addition of a 200 μ M dose of SC-59840 to *NMT/nmtΔ* cells produces no detectable inhibition of Arf N-myristoylation (Fig. 5).

DISCUSSION

The results presented above indicate that it is possible to monitor the state of cellular protein N-myristoylation in fungi using a simple assay based on differences in the mobilities of myristoylated and nonmyristoylated forms of Arfs. This protein mobility shift assay was used to help establish that inhibition of *C. albicans* Nmt by a competitive tripeptide inhibitor produces growth arrest. Such a finding provides further support for the notion that Nmt is an attractive target for fungistatic and possibly fungicidal therapy.

Although the structural basis for the different mobilities of N-myristoylated and nonmyristoylated Arfs during SDS-PAGE is unknown, it is not unprecedented. Gpa1p is an N-myristoylated α -subunit of the heterotrimeric G

Fig. 6. SC-59383 has a fungistatic effect on *C. albicans* strain B311. (a) Cells were treated with a single dose of 100 µM SC-59383 (Δ), its enantiomer SC-59840 (□), which does not inhibit the purified Nmt from this strain *in vitro*, or vehicle alone (○; 0.5% DMSO). Growth was monitored in YNB at 30 °C. The mean of triplicate determinations at each time-point is plotted (standard deviations are not shown since values varied by < 10% among triplicates). (b) An experiment analogous to that shown in (a) except that a 100 µM dose of each compound was administered every 12 h (control, ○, = equivalent amount of DMSO vehicle). Aliquots were removed at the various time-points and c.f.u. ml⁻¹ was determined under permissive growth conditions. The results indicated that SC-59383 had a fungistatic but no detectable fungicidal effect. Symbols are as in (a). (c) Dose-response curves for the peptidomimetics. A single dose of drug at the indicated concentration was added to exponential-phase cultures of strain B311 and the OD₄₉₀ was measured 24 h later. Note that SC-59840 has no effect on growth at 1000 µM, which is at the limit of its solubility. Δ, SC-59383; □, SC-59840.

protein involved in the mating factor signal transduction cascade of *S. cerevisiae*. Dohlman *et al.* (1993) reported that α -factor treatment of MATa strains promotes replacement of a pool of nonmyristoylated Gpa1p with newly synthesized myristoyl-Gpa1p and suggested that this represents a mechanism for long-term regulation of the mating factor signalling pathway. Like the non-myristoylated form of Arf, the nonmyristoylated form of Gpa1p has a slower mobility during SDS-PAGE than the *N*-myristoylated species.

Protein *N*-myristoylation is a co-translational modification (reviewed in Johnson *et al.*, 1994a). Gpa1p is the only *S. cerevisiae* *N*-myristoylated protein that is detectable in both *N*-myristoylated and nonmyristoylated forms in cells with a wild-type NMT1 allele. The studies of Dohlman and co-workers (1993) indicated that both forms of Gpa1p are rapidly degraded, that they both need continuous protein synthesis to be maintained, and that the nonmyristoylated species was not generated by demyristoylation of *N*-myristoylated Gpa1p. Although the mechanism is not known, it appears that the Gpa1p produced after α -factor treatment of MATa NMT1 cells is more efficiently *N*-myristoylated.

Unlike the case of Gpa1p, the mechanism underlying the appearance of nonmyristoylated Arfs appears to be directly attributable to a reduction in Nmt activity. In cells with wild-type NMT alleles, Arfs are fully *N*-myristoylated, i.e. this substrate for Nmt does not 'escape' covalent *N*-myristoylation. When Nmt activity is diminished, either through genetic or pharmacological manipulations, an 'escape' from *N*-myristoylation is observed. The escape increases in a progressive fashion as the temperature-sensitive Nmt mutant is subjected to higher incubation temperatures. Escape can be

suppressed when this mutant temperature-sensitive Nmt with reduced affinity for myristoyl-CoA is exposed to augmented cellular myristoyl-CoA pools. Thus, several factors make the Arf proteins very attractive reporters for monitoring cellular Nmt activity. They are fully *N*-myristoylated in cells with fully active Nmt (i.e. the baseline appears to be 100% *N*-myristoylation). Their gel mobility shifts are readily detected because they are small proteins and both *N*-myristoylated and non-myristoylated forms have similar reactivity to antibodies. Finally, because Arfs are ubiquitous proteins that are highly conserved throughout evolution, we anticipate that the mobility shift assay can be applied to a wide variety of organisms.

As noted in the Introduction, Arf1p and Arf2p are interchangeable proteins that need myristate to fulfill their essential functions. In *S. cerevisiae*, Arf1p is the predominant cellular Arf, accounting for ~90% of total cellular Arfs (Stearns *et al.*, 1990). Although the loss of *N*-myristoylated forms of Arfs is a marker of growth arrest, several observations suggest that it is unlikely to be the only *N*-myristoylprotein whose loss contributes to the demise of cells. Overexpression of ARF1 is not sufficient to rescue *nmt1-451D* cells (Johnson *et al.*, 1993). NMT1 *arf1Δ* ARF2 cells are viable even though their levels of Arfs are ~10% those in isogenic ARF1 ARF2 cells. An *nmt1-451D* ARF1 ARF2 cell has more *N*-myristoylated Arf1p/Arf2p 2–4 h after growth arrest than does a proliferating NMT1 *arf1Δ* ARF2 cell (i.e. 50% compared to 10% of levels encountered in a wild-type NMT1 ARF1 ARF2 cell). Finally, *S. cerevisiae* contains other *N*-myristoylproteins such as Gpa1p which are essential and which require myristate for their function.

In *C. albicans*, a $\geq 50\%$ reduction in N-myristoylated Arf appears to be a marker of an unviable cell. Genetic or pharmacological manipulations of Nmt activity that reduce the fractional representation of N-myristoylated Arfs to this value would be expected to have a fungicidal effect. Other N-myristoylproteins are known to be produced by this organism, including a homologue of Gpa1p encoded by the *CAG1* gene (Sadhu *et al.*, 1992). However, there are no published genetic or biochemical studies about whether the fungal Arfs or the Gpa1p homologue are essential and/or dependent upon myristate for function.

We were able to conclude that the fungistatic effects of SC-59383 are directly related to its inhibition of cellular Nmt. This conclusion is based, in part, on the effects of the active tripeptide inhibitor of *C. albicans* Nmt (SC-59383) on Arf N-myristoylation and growth. Equally important was the fact that its enantiomer, SC-59840, had no effect on Nmt activity *in vitro*, on growth of *C. albicans* in culture, or on Arf N-myristoylation *in vivo*. Together, these findings indicated that the fungistatic activity of SC-59383 is not due to a 'non-specific' physical (nonenzymic) effect on the organism (e.g. a detergent effect).

The inhibition of Arf N-myristoylation produced by SC-59383 is modest and well below the value of 50% associated with lethality. Such a modest reduction is consistent with its fungistatic rather than fungicidal effect. The biological effects of SC-59383 may be limited by its susceptibility to degradation by cellular carboxypeptidases: although prolonged incubation of the compound with media harvested from *C. albicans* cultures produces no degradation, addition of cell lysates results in removal of the C-terminal cyclohexyl-L-alanine or this residue and L-lysine (data not shown). Repeated additions of SC-59383 to cultures produces a more prolonged growth suppressive effect, consistent with the notion that the compound has limited stability within cells. It will be interesting to examine the effects of modifications in SC-59383 that make it less susceptible to carboxypeptidase digestion: greater stability may allow a related compound to produce a more pronounced inhibition of cellular protein N-myristoylation and a fungicidal effect.

Finally, it is important to note that SC-59383 retains the Ser-Lys dipeptide critical for recognition of natural substrate proteins by fungal Nmts (reviewed in Johnson *et al.*, 1994a). Thus, it appears unlikely that *C. albicans* Nmt will be able to acquire mutations that would allow it to escape the growth arrest produced by Ser-Lys-containing peptidomimetic inhibitors without compromising the ability of this essential enzyme to fulfill its function *in vivo*.

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