Short communication

BREFELDIN A DECREASES THE ACTIVITY OF THE GENERAL AMINO ACID PERMEASE (GAP1) AND THE MORE SPECIFIC SYSTEMS FOR L-LEUCINE UPTAKE IN *Saccharomyces cerevisiae*

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Abstract: Brefeldin A is a commonly used antifungal agent that reversibly blocks protein transport from the endoplasmic reticulum to the Golgi complex. In this study, we aimed to characterize L-leucine uptake in *Saccharomyces cerevisiae* in the presence of brefeldin A. For this purpose, we used a synthetic medium, containing L-proline and the detergent SDS, which allows the agent to permeate into the yeast cell. The results obtained with a wild type strain and a *gap1* mutant indicate that BFA causes either direct or indirect modification of the transport and/or processing of L-leucine permeases. The presence of BFA affects the kinetic parameter values for L-leucine uptake and decreases not only the uptake mediated by the general system (GAP1), but also that through the specific BAP2 (S1) and/or S2 systems.

Key words: Brefeldin A, Leucine permeases, *Saccharomyces cerevisiae*

INTRODUCTION

Amino acid transport from the extra-cellular medium in the yeast *Saccharomyces cerevisiae* is mediated by the general amino acid permease (GAP1) and various other permeases with more restricted specificities [1, 2]. GAP1 catalyzes the transport of all the D- and L-isomers of the amino acids normally found in proteins. It is induced by growth on a nitrogen-poor source,

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implying that it is involved in the acquisition of amino acids for use by cells as a source of nitrogen [1].

BAP2 (S1) [3] and S2 are group-specific systems which transport all three branched-chain amino acids (leucine, valine and isoleucine). S1 has a high affinity for the substrate, but transports at a relatively low velocity, while S2 has a low affinitiy and a high velocity. Although S1 and S2 can operate in the presence of NH_4^+, their activities are strongly decreased when cells are grown in a medium containing this ion [4]. Three different permeases are involved in L-leucine uptake: the general amino acid permease (GAP1), and the more specific BAP2 (S1) and S2 systems. The comparative contribution of each permease depends on the nitrogen source in the culture medium [1]. All of these amino acid permeases (AAPs), like most of the plasma membrane proteins, proceed through the secretory pathway from the endoplasmic reticulum (ER), across the Golgi Complex (GC), until they reach the plasma membrane [5]. There is a growing body of evidence about the regulation of amino acid permease activity at the level of protein transport through the ER-Golgi system [6-8].

Differential permease activity is subject to control by both exocytic and endocytic trafficking processes. However, only limited information has been obtained concerning the fate of most amino acid permeases in the later stages of the protein trafficking process which determines targeting to the plasma membrane. For example, Umebayashi and Nakano found that localization of Tat2p, the high-affinity tryptophan permease, requires ergosterol and is controlled by the external tryptophan concentration [9].

Brefeldin A (BFA) is an antifungal agent that reversibly blocks protein transport from the ER to the GC [10-12]. This effect is very useful in the study of the protein traffic along the different compartments of the secretory route.

To date, there has been no report on the effect of this drug on the different transport systems of nutrients in *S. cerevisiae*. However, for CHO-K1 cells, it has been reported that complex glycosphingolipids containing sialic acid reach the plasma membrane through a pathway that is not sensitive to the action of BFA [13]. Although an *erg6* mutation increases the permeability of *S. cerevisiae* to many different drugs, including BFA, the transport and processing of amino acid permeases are nevertheless affected in these mutants [9]. For this reason, in a previous study, we performed a protocol that allowed BFA to permeate into the yeast wild type strain MMY2 [14] when its cells were incubated in a medium containing L-proline as the only nitrogen source, and a low concentration of SDS. A feature of this technique is that it does not require mutations in ergosterol biosynthesis. We obtained yeast cells sensitive to BFA but without the altered permease transport and processing introduced by the *erg6* mutation.

Taking this into consideration, in this study, we aimed to characterize the kinetics of L-leucine uptake in *S. cerevisiae* in the presence of BFA, by using the above incubation conditions with an increased permeability to BFA. We studied the kinetics of the process in the wild type strain MMY2 and in its derived

mutant *gap1* MMY2/H3 [4], which lacks general system activity. We also evaluated the kinetic parameters for the uptake of L-citruline, an amino acid that is only transported by the general system (GAP1) [15].

MATERIALS AND METHODS

Strains and growth conditions

Saccharomyces cerevisiae MMY2 (MAT a *ura3*) and its derived strain MMY2/H3 (*MAT* a *ura3 gap1*) [13] were used. Cells were grown at 30ºC with continuous shaking in the culture medium indicated for each assay.

Culture media and chemicals

1. MPM (minimal proline medium): 0.17% w/v yeast nitrogen base (Difco, catalogue number 0335-15-9); 0.1% w/v L-proline; 2% w/v dextrose.
2. YPD: 1% yeast extract, 1% peptone, 2% dextrose.When colony counting was required, 2% agar was added to prepare a YPD solid medium.

The MPM was supplemented with 20 mg/l uracil. In order to increase cell permeability to BFA, SDS was added to the medium to a final concentration of 3×10^{-3}% (w/v) as indicated below under "Cell culture and BFA treatment".

The Optical Density (OD) of each yeast cell suspension was determined at 570 nm and used to calculate the number of cells/ml. In our conditions, 1.0×10^{7} cells/ml is equal to 1 mg dry weight (dw) of yeast per ml. BFA stock solution: 7.7 mg/ml BFA in 68:32 ethanol:DMSO.

Cell culture and BFA treatment

Twenty-five milliliters of YPD medium inoculated at an initial concentration of 1×10^{6} cells/ml of the MMY2 or MMY2/H3 strains were cultured overnight at 30ºC with continuous shaking. After 17 hours of incubation, the cells were centrifuged at 4000 rpm using a clinical centrifuge, and the cell pellets were washed once with distilled water. Twenty-five milligrams (wet weight) of cells were re-suspended in 25 ml of MPM containing SDS at a final concentration of 3×10^{-3}%. An equal quantity of cells was re-suspended in the same medium but containing 40 μg/ml BFA.

Both cultures were incubated with shaking at 30ºC for 60 min. Next, a 100-μl aliquot of each culture was collected to inoculate plates containing solid YPD medium in order to determine viability by colony counting. The remaining suspensions were centrifuged; pellets were washed once and re-suspended in distilled water to a final concentration of 6.3 mg (dw) of cells/ml.

L-^{14}C-amino acid uptake assay

640-μl aliquots containing 4 mg of MMY2 or MMY2/H3 cells grown and incubated as indicated above were mixed with 80 μl of L-^{14}C leucine or L-^{14}C-citrulline (5 mCi mmol^{-1}) and 80 μl of 200 mM potassium phthalate buffer (PHK), pH 4.5. The mixture was incubated at 30ºC, and 200-μl aliquots were collected at different times. Every sample was filtered through 25-mm diameter

glass-fibre filters (Schleicher & Schuell, Inc, NH, USA), and washed with three volumes of cold 20 mM PHK. The radioactivity on each filter was measured with a liquid scintillation counter (Model 1214 Rackbeta). Every measurement is the average of two experiments ($P < 0.05$).

Proton extrusion assay

This assay was carried out by adding 5 or 10 mM glucose to a yeast suspension of 12-15 mg (dw) in 10 ml of 4 mM PHK. In order to determine the effect of BFA, this drug was added together with the D-glucose at a final concentration of 50 μg/ml.

Changes in the pH values were measured with a Corning 130 pH meter. A previous calibration with pulses of a known concentration of HCl was performed to calculate the nmoles of H^+ extruded.

RESULTS AND DISCUSSION

L- leucine uptake

The effect of BFA on L-leucine uptake was measured on cells incubated in MPM with SDS for 60 min either with or without the addition of BFA. Samples containing 5 mg dw/ml (final cell concentration) of MMY2 cells were then incubated with L-^{14}C-leucine and aliquots were collected at 0, 1.5 and 3 min.

The initial velocity for each amino acid concentration was calculated using a linear regression program with the values of L-^{14}C-leucine in $\mu mol/g_{dw}$ normalized according to cell viability [14].

The V vs [S] of L-leucine uptake for the wild type strain MMY2 is represented in Fig. 1. The values of the initial velocity decreased when the cells were incubated with BFA and concentrations of external L-leucine up to 2 mM. This confirms the results of our previous study, in which only one external amino acid concentration was used [14].

In order to compare the values of the kinetic parameters obtained under different conditions, we performed a data adjustment using Sigmaplot Version 7.0. (Tab. 1). The parameters were calculated according to the equation $v = V_{max} \times [S] / (K_T + [S])$. This relationship was adequately described by the analogous Michaelis-Menten equation by assuming the presence of a single, saturable process. v is the rate of substrate (in this case, L-leucine or L-citrulline) transport from a substrate concentration of [S]; V_{max} is the maximal rate of mediated transport when the carrier is saturated with the substrate; and K_T is the apparent Michaelis constant. Although the values for maximal velocity (V_{max}) obtained with BFA-treated and untreated cells were similar, the increase in the K_T value in the BFA-treated cells indicates that they would require a higher L-leucine concentration to reach that velocity. The higher K_T value might be due to a decrease in the activity of a) the General System, b) more specific systems, or c) all of the systems involved in L-leucine uptake.
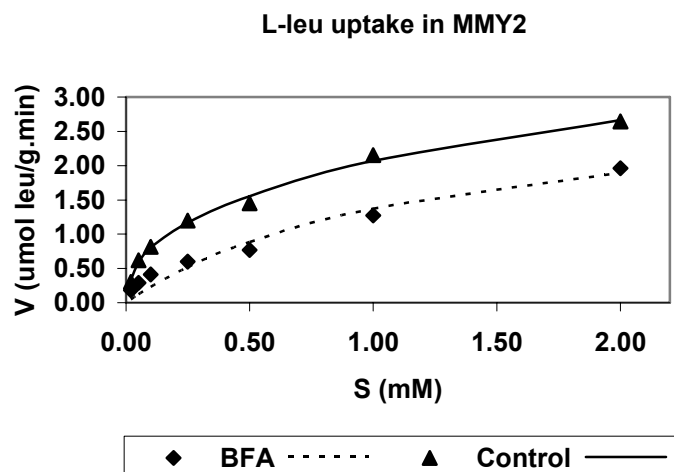
L-leu uptake in MMY2

Fig. 1. The V vs [S] of L-leucine for the wild type strain MMY2. Cells in MPM medium were incubated with 3×10^{-3}% SDS (final concentration) for 60 min either with or without 40 μg/ml BFA. 5 mg/ml samples of MMY2 cells were then incubated with L-^{14}C-leucine (5 mCi mmol^{-1}), and aliquots were collected at 0, 1.5 and 3 min. The initial velocity for every amino acid concentration was calculated using a linear regression programme with values of L-^{14}C-leucine in μmol/g_{dw} normalized according to cell viability.
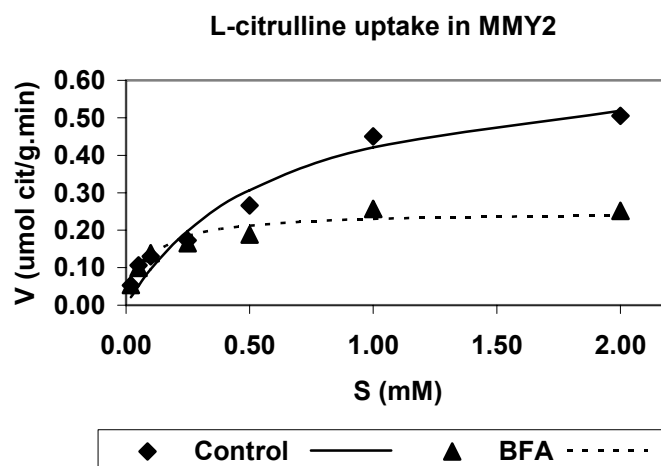
L-citrulline uptake in MMY2

Fig. 2. The V vs [S] of L-citruline for the wild type strain MMY2. Cells in MPM medium were incubated with 3×10^{-3}% SDS (final concentration) for 60 min either with or without 40 μg/ml BFA. Samples of 5 mg/ml of MMY2 cells were then incubated with L-^{14}C-citruline (5 mCi mmol^{-1}) and aliquots were collected at 0, 1.5 and 3 min. The initial velocity for every amino acid concentration was calculated using a linear regression programme with values of L-^{14}C-leucine in μmol/g_{dw} normalized according to cell viability.

Tab. 1. L-leucine and L-citrulline uptake parameters for the strains.

Strain	Amino acid	K_T (mM)	V_{max} (µmol/g.min)
MMY2	L-leucine	0.34 ± 0.07	2.93 ± 0.73
MMY2 + BFA	L-leucine	1.23 ± 0.32	3.06 ± 0.66
MMY2	L-citrulline	0.60 ± 0.14	0.68 ± 0.12
MMY2 + BFA	L-citrulline	0.09 ± 0.02	0.25 ± 0.05
MMY2/H3	L-leucine	2.46 ± 0.37	1.77 ± 0.34
MMY2/H3 + BFA	L-leucine	0.80 ± 0.16	1.09 ± 0.21

The values of the K_T and V_{max} of every experiment were calculated according to the equation $v = V_{max} \times [S] / (K_T + [S])$. The data was adjusted using Sigmaplot Version 7.0. v, uptake velocity; V_{max}, maximal uptake velocity; [S], amino acid concentration; K_T, uptake constant. Standard error values ($\pm$ S.E.) were generated by the program in order to fit the parameters to the experimental data.

L-citrulline uptake
L-citrulline uptake was measured under the same conditions as used for L-leucine uptake. In this case, the values of the initial uptake velocity at various external amino acid concentrations were decreased by BFA treatment. In fact, no significant increase in velocity was seen above 1.0 mM L-citrulline (Fig. 2). The calculated parameters indicated a decrease in the maximal velocity for Gap1p. This observation allowed us to infer that the low rate of uptake in the wild type MMY2 strain is due to a decrease in the activity of the General System, although a concomitant decrease in the activity of more specific systems could not be dismissed.
In order to investigate this possibility, we determined the kinetics of L-leucine uptake in the MMY2/H3 strain that lacks General System activity. The data adjustment was performed analogously to that for the wild type strain, assuming the same model ($v = V_{max} \times [S] / (K_T + [S])$) for amino acid uptake. Although the two specific permeases (S1 and S2) carry out L-leucine uptake in *gap1* mutants [13], we were not able to distinguish them after the BFA incubation. The correlation coefficient obtained fits in such a way that they can only be considered one system; this allows us to compare the L-leucine uptake in the wild type and *gap1* mutant strain. The results presented in Tab. 1 show that the maximum velocity decreased by nearly 50% upon BFA incubation, although an increase in the affinity of the system for L-citrulline in MMY2 and L-leucine in MMY2/H3 is also revealed by the adjustment. Considering that in both the wild type strain and the *gap1* mutant, more than one system for L-leucine uptake may be working, it is possible to assume that BFA might affect a low affinity system or factor involved in the process while the uptake was held by a system with less velocity but more affinity.

Proton extrusion measurements

Another important factor determining amino acid uptake is the plasma membrane H^+-ATPase activity, which is responsible for generating a gradient of protons across the plasma membrane. Amino acid transport systems use this gradient to drive amino acid accumulation. H^+-ATPase trafficking up to the plasma membrane takes place in COPII vesicles, which may be altered by treatment with BFA [16]. The decreases observed in our conditions for L-leucine and L-citruline uptake could be due to the incapability of cells to generate an appropriate proton gradient. Serrano *et al.* [17] demonstrated that a decrease in proton pumping may be correlated with a decrease in the amino acid uptake. Both our wild type strain and its *gap1* mutant showed values of 45-50 nmoles of H^+ 10 min after glucose was added to the medium. No significant differences were observed when BFA was added.

Our results indicate that BFA causes either a direct or indirect modification to the transport and/or processing of L-leucine permeases, which affect the kinetic parameter values. BFA treatment decreases the leucine uptake mediated by the general system GAP1 and the specific systems BAP2 (S1) and/or S2. Considering the turnover of permeases and the BFA-mediated blocking of ER to GC transport, which provides newly synthesized proteins to the plasma membrane, the kinetic alteration observed may be due to a decrease in the abundance of Gap1 and/or Bap2 proteins at the cell surface. On the other hand, we could not rule out alterations in any factor of transport and/or processing that could be common to different transport systems.

Our current work is focused on characterizing the kinetics of the different L-leucine specific permeases in mutant strains resistant to BFA in order to begin exploring if each of these individual AAPs follows a separate sorting pathway to the PM.

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