FEBS Letters 509 (2001) 370–374 FEBS 25582

Characterisation of a concentrative type of adenosine transporter from *Arabidopsis thaliana* (ENT1,*At*)

Torsten Möhlmann, Zeina Mezher, Gabriele Schwerdtfeger, H. Ekkehard Neuhaus*

Universität Kaiserslautern, Pflanzenphysiologie, Postfach 3049, D-67653 Kaiserslautern, Germany

Received 23 August 2001; revised 30 October 2001; accepted 12 November 2001

First published online 22 November 2001

Edited by Marc Van Montagu

Abstract Here we report on the isolation of an *Arabidopsis thaliana* cDNA that is able to complement a *Saccharomyces cerevisiae* mutant unable to synthesise adenine. This cDNA encodes a highly hydrophobic protein (ENT1,*At*) of 428 amino acids, showing high similarity to the human nucleoside transporter hENT1. Yeast cells expressing ENT1,*At* are able to grow on adenosine-containing media, adenosine import exhibited an apparent affinity (K_M) of 3.6 μM, and led to accumulation of this nucleoside within the yeast cell. Transport is inhibited by various nucleosides. Typical inhibitors of ENT-type nucleoside transporters do not inhibit ^{3}H-adenosine import. The presence of protonophores abolished adenosine import, indicating that ENT1,*At* catalyse a proton-dependent adenosine transport. This is the first functional characterisation of a plant nucleoside transport protein.

Key words: Nucleoside transport; Adenosine; Yeast; *Arabidopsis thaliana*

1. Introduction

Nucleoside transport across human, animal, fungi and bacterial cell membranes has been intensively characterised in the past at the physiological and molecular level [1–4]. The corresponding transport proteins mediating nucleoside transport are classified into two groups: one group catalyses facilitated diffusion along a concentration gradient (ENT, equilibrative nucleoside transporters) [5], whereas the members of the other group (CNT, concentrative nucleoside transporters) are able to transport nucleosides against an existing concentration gradient [6]. Thus, the activity of the latter requires metabolic energy and up to now both sodium or proton coupled nucleoside transport catalysed by CNT-type carriers have been identified [6,7].

The function of nucleoside import into animal cells is mainly to allow nucleotide synthesis via salvage pathways in cells lacking the capacity for de novo synthesis [1,8,9]. Moreover, adenosine functions as an extracellular messenger molecule [2], and is known to cause cell toxicity and immunosuppression, to influence cell morphology, to act as a vasodilator, and to stimulate hormone secretion [10]. Therefore, transport of these compounds plays an important role in the regulation of cellular signalling and the regulation of metabolism and development.

In contrast, our knowledge about the function of nucleosides in plant cells and tissues is much more limited. In general, plant cells harbour enzymes allowing nucleoside salvage and some of the genes coding for these enzymes have been identified at the molecular level [11,12]. In plants, nucleoside uptake into the phloem tissue has been detected [13]. The phloem represents a tubular system, consisting of living cells, which allows long distance transport of organic products between plant organs [14]. These products are either generated by photosynthesis, or liberated by degradation of various storage products [14]. Cotyledons prepared from developing castor-bean seedlings import nucleosides, which have previously been liberated from mobilising endosperm tissue [13]. In addition, intact petunia pollen cells are able to import exogenous nucleosides via both an active import and a facilitated diffusion [15,16]. Recently, a cDNA from *Arabidopsis thaliana* coding for a protein with substantial similarity to the human equilibrative nucleoside transporter 1 (hENT1) has been described [17]. However, a proof of function of this protein is still lacking [17]. Thus, the transport proteins involved in the loading of nucleosides into phloem, or involved in nucleoside uptake into other types of plant cells are still unknown. Therefore, we set up an experimental approach to identify and characterise plant nucleoside transporters at both the molecular and biochemical level.

We have used a *Saccharomyces cerevisiae* mutant, unable to synthesise adenine, in an attempt to identify a cDNA in an *A. thaliana* expression library coding for a nucleoside transport protein. Yeast mutants have previously been shown to represent an extremely powerful experimental tool allowing the identification of a wide range of homologous or heterologous transport proteins. This approach was effective in the identification and characterisation of a number of plant membrane proteins that catalyse the transport of inorganic ions, or organic molecules across the plant plasma membrane [18]. Recently, using this *S. cerevisiae* mutant a nucleoside transport protein from the parasitic protozoan *Trypanosoma brucei* has been identified [19].

2. Materials and methods

2.1. Enzymes and biochemicals

Restriction endonucleases and DNA-modifying enzymes were purchased from Boehringer-Mannheim (Mannheim, Germany). Radioactively labelled nucleosides were purchased from NEN. Amino acids to

*Corresponding author. Fax: (49)-631-205 2600.
E-mail address: neuhaus@rhrk.uni-kl.de (H.E. Neuhaus).

Abbreviations: NBMPR, nitrobenzylmercaptopurine; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone

PII: S0014-5793(01)03195-7

supplement auxotrophies were from Sigma (Deisenhofen, Germany). Yeast nitrogen base without amino acids was from Remel (distributed by Creatogen Biosciences, Augsburg, Germany).

2.2. Strains and growth conditions

For functional complementation the *S. cerevisiae* strain W303 (Mat α, leu2-3, leu2-112, trp1-1, ura3-1, his3-11, his3-15, ade2-1, can1-100) was used [20]. Growth of yeast under non-selective conditions was performed in YPD medium (1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose). Yeast cells were grown at 30°C either in liquid culture with vigorous shaking, or on agar plates (media supplemented with 20 g agar/l). *Escherichia coli* cells (XL1Blue, Stratagene, Heidelberg, Germany) were grown in YT medium (0.8% peptone, 0.5% yeast extract, and 0.25% NaCl) with or without ampicillin (200 mg/l) and tetracycline (10 mg/l).

2.3. Preparation of library DNA and yeast transformation

The cDNA expression library derived from whole *A. thaliana* seedlings in the yeast expression vector pFL61 [21] was a kind gift from Dr. Michèle Minet (CNRS, Gif-sur-Yvette, France). The library was amplified as explained before [22]. 1 μg of library plasmid was used for yeast transformation according to a standard protocol [23]. Out of 10^4 transformants selected on synthetic minimal media lacking uracil and adenine but containing 150 μM adenosine, one colony grew. The library plasmid was isolated as given in [24] and retransformed into *E. coli*. For sequencing of the ENT1,*At* cDNA from plasmid pTM86, the oligonucleotides 5′-GTTTTCAAGTTCTTAGATGC-3′, reading from the PGK promotor towards the insert and 5′-AGCGTAAAG-GATGGGG-3′ reading from the PGK terminator towards the insert [21] were used. Sequencing was performed by 'Seqlab' Sequencing Laboratories, (Göttingen, Germany).

2.4. Plasmids and DNA constructions

As a control in uptake experiments, plasmid pTM88, harbouring the ENT1,*At* cDNA in antisense orientation in respect to the PGK promotor, was generated. The complete coding region of the ENT1,*At* cDNA was amplified by polymerase chain reaction (PCR) with pfu-DNA polymerase using primers TM41 (5′-CAAATGACCACCACC-GATAAATC-3′) and TM42 (5′-GAATCAAATGACCCAGAAC-CAAG-3′). After filling in the *Not*I digested restriction sites of vector pfl61, the PCR product was inserted in the correct orientation as checked by restriction digestion.

2.5. Transport experiments

For transport experiments, yeast cells were retransformed with plasmid pTM86. Yeast cells were grown in minimal media containing adenine, but lacking uracil. Cells were grown to an OD_{600} of 0.5 to 1.5, harvested, washed twice with 25 mM $NaPO_4$ buffer medium (pH 6.0) and resuspended in the same buffer medium resulting in a final OD_{600} of 10. Cells were kept on ice until transport experiments were initiated. 100 μl of cells were added to the same volume of transport medium containing [^{3}H]adenosine in $NaPO_4$ buffer medium (if not indicated otherwise), and incubated at 30°C. At the end of the incubation, cells were filtered on membrane filters (Supor membrane disc filters, 0.45 μm pore size, Pall Gelman Laboratory, USA), previously set under vacuum.

3. Results

3.1. Complementation of a S. cerevisiae mutant unable to synthesise adenine

The *S. cerevisiae* strain mutant W303 is not able to synthesise adenine. Therefore, to grow this mutant, adenine has either to be present in the medium (Fig. 1A), or a transport protein has to catalyse adenosine import, which subsequently is converted in the yeast cell to adenine. After transformation of the mutated yeast cells with an *A. thaliana* cDNA library [21] we identified one yeast colony able to grow on minimal media containing adenosine (Fig. 1B). Wild-type cells, as well as cells harbouring the control plasmid pTM88 grow on adenine-containing medium, but not on medium supplemented with adenosine (Fig. 1B).

Sequence analysis of the plasmid (pTM86) isolated from the transformed cells revealed that the cDNA insert (1596 bp) was identical to an *A. thaliana* genomic sequence (AAC18807; PID g3176684), and contained a 36 bp untranslated region at the 5′-end, and 273 bp untranslated region at the 3′-end. Recently, a similar cDNA was isolated but the deduced amino acid sequence differed in two positions, namely at amino acid position 89 a proline instead of a leucine residue was determined, and at amino acid position 410 a threonine instead of a methionine residue was identified [17]. However, the authors sequenced a PCR product [17] and it may be that failure function of the polymerase caused these discrepancies.

The cDNA identified by us encodes a highly hydrophobic protein of 428 amino acids, exhibiting 11 predicted membrane spanning segments (analysis according to [25]). The deduced

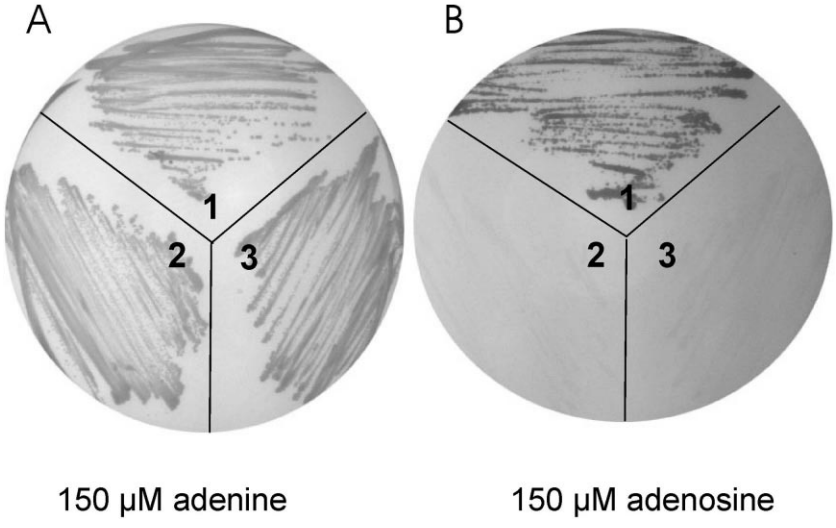

Fig. 1. Adenosine utilisation in *S. cerevisiae*. A: Growing yeast cells harbouring ENT1,*At* (1), control vector (2), or wild-type yeast cells (3) on agar containing 150 μM adenine. B: Growing yeast cells harbouring ENT1,*At* (1), control vector (2), or wild-type yeast cells (3) grown on minimal media containing 150 μM adenosine.

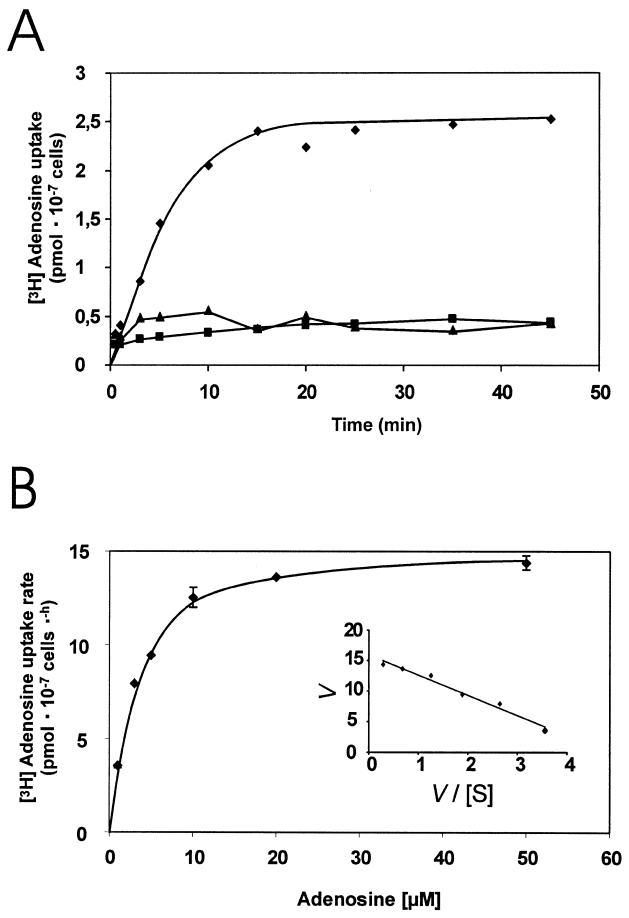

Fig. 2. Transport kinetics of [^{3}H]adenosine import in intact yeast cells. A: Time dependency of adenosine transport. Yeast cells harbouring ENT1,*At* were at a [^{3}H]adenosine concentration of 1.8 μM. ◆, cells harbouring ENT1,*At*; ■, a control vector; ▲, adenosine transport by cells harbouring ENT1,*At* in the presence of 5μM CCCP. B: Michaelis–Menten kinetics of adenosine import. Cells were incubated for 7 min in incubation medium containing the indicated concentrations of [^{3}H]adenosine. Inset: Eadie–Hofstee plot of the data indicating an apparent affinity of 3.6 μM. Uptake rates into control cells has been subtracted. Data are the mean of three independent experiments. Standard error of the mean less than 4% of the given value.

protein shares 24% identical amino acids to both the hENT1 and the equilibrative nucleoside transporter mENT3 from mouse. Therefore, we named the protein ENT1,*At*. The ENT1,*At* protein also exhibits high sequence similarity to 7 other *A. thaliana* proteins that have also not been functionally characterised so far. These sequences have the following NCB accession numbers and the deduced proteins exhibit following degrees of identity: AAG10625 exhibiting an identity of 45%, CAB81054 exhibiting an identity of 28%, AAF04424 exhibiting an identity of 28%, CAB81053 exhibiting an identity of 26%, CAB81055 exhibiting an identity of 26%, CAB81056 exhibiting an identity of 26%, and AAD25545 exhibiting an identity of 26%.

3.2. Transport characteristics of ENT1,At

To characterise the function of ENT1,*At* we first analysed the time dependency of [^{3}H]adenosine uptake. Adenosine import into yeast cells harbouring plasmid pTM86 was linear with time for about 10 min and then reached saturation at about 2.5 pmol adenosine/10^7 cells (Fig. 2A; we checked in further control experiments, that uptake was linear for about 10 min also at adenosine concentrations of up to 50 μM, data not shown). Assuming a yeast cell volume of 1 μl/3×10^7 cells, the externally given ^{3}H-adenosine (1.8 μM) has been concentrated by a factor of about 4.2. Yeast cells harbouring the control plasmid pTM88 showed almost no adenosine import over a time span of up to 45 min (Fig. 2A). The same holds true for wild-type yeast cells (data not shown). Uptake of adenosine displayed typical Michaelis–Menten-type kinetics (Fig. 2B) and an apparent affinity (K_M) of 3.6 μM (inset Fig. 2B).

To analyse whether ENT1,*At* functions as an equilibrative, or as a concentrative adenosine transporter we added the uncoupler carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP; 5 μM final concentration) during uptake. As demonstrated in Fig. 2A, CCCP completely abolished adenosine uptake, which is consistent with the strong inhibitory effect of dinitrophenol (DNP) on adenosine import catalysed by ENT1,*At* (Table 1). Replacement of sodium phosphate by potassium phosphate in

Table 1
Inhibition of ENT1,*At* mediated [^{3}H]adenosine uptake in intact yeast cells by nucleotides, nucleobases and related substances

Additive	[^{3}H]Adenosine uptake (% of control)
None	100
Nucleosides:	
Adenosine	26
Cytidine	53
Guanosine	76
Uridine	93
Inosine	53
2′-Deoxyadenosine	21
2′-Deoxycytidine	42
2′-Deoxyguanosine	59
2′-Deoxythymidine	72
2′-Deoxyinosine	71
Nucleobases:	
Adenine	90
Cytosine	107
Guanine	91
Thymine	101
Uracil	117
Hypoxanthine	112
Nucleotides:	
dATP	91
ATP	95
ADP	99
AMP	99
CTP	87
GTP	91
UTP	97
NAD	97
Inhibitors:	
Dilazep (20 nM)	105
Dilazep (20 μM)	105
Dipyridamole (20 nM)	115
Dipyridamole (20 μM)	98
NBMPR (20 nM)	75
NBMPR (20 μM)	67
DNP (1 mM)	4

[^{3}H]Adenosine uptake was performed at a substrate concentration of 1.8 μM for 7 min. Inhibitor concentrations were 18 μM (if not stated otherwise). Data are the mean of three independent experiments. Standard error of the mean less than 4% of the given value.

the incubation medium does not inhibit adenosine import (data not shown). Furthermore, we analysed the effect of typical inhibitors of mammalian ENTs such as nitrobenzylmercaptopurine (NBMPR), dilazep, and dipyridamole [1]. NBMPR given at a high concentration (20 nM) inhibited adenosine import by about 25%, whereas both dilazep, and dipyridamole did not affect transport activity of ENT1,*At* (Table 1).

To assess the substrate specificity of ENT1,*At* in more detail we examined the effect of structurally related compounds on adenosine import. Unlabelled adenosine, and deoxyadenosine showed the highest inhibition, followed by inosine, cytidine and the corresponding 2′-deoxynucleosides. Guanosine exerted little effect on adenosine transport, all other substances tested had no effect (Table 1). IC_{50} values were determined at a substrate concentration of 3.6 μM adenosine for 2-deoxyadenosine (4 μM), inosine (20 μM), cytidine (20 μM) and guanosine (28 μM). Thus ENT1,*At* represents a high affinity adenosine transporter which also exhibits affinity to other nucleosides.

4. Discussion

ENT1,*At* has been identified by functional complementation of the yeast mutant W303 and represents the first plant nucleoside transport protein described at the biochemical level. This transporter exhibits a high affinity to adenosine (3.6 μM; Fig. 2B) and is inhibited to 21–76% residual activity by both purine and pyrimidine nucleosides, as well as by 2′-deoxynucleosides (Table 1). Thus ENT1,*At* is most likely a transport protein with broad substrate specificity. The inhibitory effect of various nucleosides on adenosine transport by ENT1,*At* (Table 1) is in accordance with the biochemical properties of other nucleoside transporters. Members of both the CNT- and the ENT-type mammalian nucleoside transport proteins transport more than one nucleoside [1]. Interestingly, uridine did not substantially inhibit adenosine transport by ENT1,*At* (Table 1). As uridine is considered to represent a universal permeant transported by all mammalian nucleoside transport proteins [6], the ENT1,*At* protein differs in this biochemical property.

Both the predicted topology of ENT1,*At* with 11 transmembrane domains, and the high sequence similarity to the known ENT-type transport proteins supported the assumption that ENT1,*At* might catalyse an equilibrative nucleoside transport [17]. However, the inhibitory effect of the protonophores CCCP and DNP (Fig. 2 and Table 1) clearly indicate that this is not the case. Obviously, the proton gradient across the yeast plasma membrane is required to mediate adenosine import into intact yeast cells. The required proton motive force to allow a concentrative nucleoside import into plant cells could be generated by a highly active ATPase, known to reside in the plasmalemma [26].

Categorisation of ENT1,*At* as a concentrative type of nucleoside transporter is further supported by the lack of effect of the inhibitors dilazep, and dipyridamole on ENT1,*At* activity (Table 1). Typically, these compounds are classified as ENT specific inhibitors, which do not affect CNT-type nucleoside transporters [6]. NBMPR given at a concentration of 20 nM decreased ENT1,*At* activity to about 75% of the corresponding control rate (Table 1). In contrast to dilazep and dipyridamole, the ribonucleoside NBMPR also inhibits concentrative-type nucleoside transporters at high concentrations. However, ENT-type transporters exhibit IC_{50} values between 5 nM and 1 μM for highly sensitive or slightly sensitive transporters, respectively [6]. To this point we cannot explain why NBMPR given at 20 μM inhibited ENT1,*At*-mediated adenosine transport only slightly stronger than at 20 nM (Table 1). Obviously, further experiments have to be carried out on adenosine transporters from plants to gain deeper insight into the biochemical properties.

Both the *A. thaliana* transport protein ENT1, and the nucleoside transporter *Tb*NT2 from *T. brucei* (*Tb*NT2) share structurally a higher similarity to ENT-like nucleoside transporters than to CNT-like nucleoside transporters (see above and [27]). However, both proteins catalyse a proton driven nucleoside transport (Fig. 1A) [27]. Therefore, it is not possible to take the predicted molecular architecture of a nucleoside transport protein as an indicator for this general biochemical feature. Interestingly, a detailed phylogenetic analysis revealed that the predicted nucleoside transporters present in the genome of *A. thaliana* cluster closer to homologous proteins from parasitic protozoa than to all other eukaryotic nucleoside transport proteins [5]. It remains to be analysed whether the plant transporters share further biochemical properties with the parasitic proteins.

The proton dependency of adenosine transport catalysed by ENT1,*At* concurs with previous measurements of purine nucleoside import into enriched petunia pollen cells [15]. Furthermore, nucleoside transport into petunia pollen cells was not sensitive to low concentrations of NBMPR [15]. Up to now it is not known whether ENT1,*At* is a pollen specific transporter and such cell specificity would contrast with previous data. Li and Wang [17] showed that ENT1,*At* mRNA accumulated to similar levels in both autotrophic and heterotrophic tissues from *A. thaliana*. Thus, it will be very interesting in the near future to analyse both the cell specificity of expression, and the biochemical properties of all eight putative nucleoside transporters from *A. thaliana* in more detail.

In general, little is known about the nucleoside levels in different plant cells and plant cell compartments. Enzymes involved in salvage reactions of purines and pyrimidines have been identified on the enzymatic and molecular level [11,12] and *A. thaliana* mutants lacking an enzyme of the nucleoside salvage pathways show non-functional pollen and thus male sterility [28]. Therefore, the possible involvement of nucleoside transport proteins in plant cell physiology will be very interesting to study in the near future. Moreover, it has been shown that nucleoside transport into pollen cells occurs by both an active and a passive transport mechanism [15,16]. Therefore, the molecular nature of the transport protein mediating passive nucleoside transport is still in question.

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